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(57) Abstract

A recombinant pox virus encoding a myelin protein is described. The recombinant pox virus can be used for generating an immune response to the myelin protein. This method involves introducing a sufficient amount of the pox virus to present the myelin protein to the immune system, expressing this myelin protein and presenting the myelin protein to the immune system.

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RECOMBINANT POX VIRUS ENCODING MYELIN PROTEIN FOR THERAPY

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BACKGROUND OF THE INVENTION

Inflammatory demyelinating diseases of the central nervous system (CNS) such as multiple sclerosis (MS) and Guillian-Barre syndrome are believed to involve an immune response against CNS autoantigens. By analogy from disease models such as experimental allergic encephalomyelitis (EAE), indirect evidence suggests that an immune response against myelin proteins, particularly myelin basic protein (MBP), plays a role in the onset and progression of inflammatory demyelination disease. For example, in humans with MS, MBP-reactive T-cells can typically be recovered from fluids of MS patients [1, 2 and 3]. These T-cells are concentrated in the cerebrospinal fluid (CSF) of MS patients when compared to controls with other neurological diseases (2). MBP-reactive T cells have also been directly detected in MS lesions (3). In EAE, MBP is a major antigen since the disease can be induced either by active immunization with MBP, antigenic fragments of MBP, or by adoptive transfer of MBP-reactive T cells (4,5).

MBP is one component of myelin protein. The MBP gene encodes several MBP-related proteins through alternative RNA splicing (see Lemki, G. (1988), Mikoshiba, K., et al. (1991), Kamholz, J., (1987)). The gene sequences of other myelin proteins such as proteolipid protein (PLP), myelin-associated glycoprotein (MAG), and

myelin/oligodendrocyte glycoprotein (MOG) are also known [see Linington, et al. Eur J. Immunol. 23: 1364 (1993).

Animals that are susceptible to EAE are recognized models for the study of human inflammatory demyelinating diseases such as, e.g., MS. EAE can typically be induced by immunization with whole myelin, MBP, antigenic fragments of MBP, or by adoptive transfer of MBP-reactive T-cells [12, 13, 4, 5]. Rodents with EAE generally recover from the disease, indicating that the immune system suppresses the disease, e.g., by providing cytotoxic T-cells or anti-inflammatory cytokines [6-11]. Human MS however, is a relapsing and remitting disease which differs from most forms of acute rodent EAE. Little is known about the factors responsible for relapses, and the role of regulatory T-cells (e.g., T suppressor cells) in the human disease has not been thoroughly investigated.

In the primate *Callithrix jacchus* (*C. jacchus*, the common marmoset), EAE can be induced by active immunization with whole white matter (WM), MBP, or by adoptive transfer of MBP-reactive-T-cells or clones [12-14]. The marmoset is an especially advantageous model for the study of inflammatory demyelinating disease, in part because siblings share a bone marrow chimerism which permits adoptive transfer of lymphocytes between genetically distinct individuals. In marmosets with EAE, T-cell and antibody reactivity against MBP is detected at the onset of disease and can be observed in peripheral blood mononuclear cell (PBMC) preparations (13).

Viruses of the family *Poxviridae* (pox viruses) are useful as vectors for the delivery of foreign genes and gene products in many clinical and research settings. Pox viruses of the genus *Orthopoxvirus*, particularly vaccinia, are used for several reasons. Among these are: (a) its wide use in humans in the eradication of smallpox; (b) its ability to infect a wide range of cells, including professional antigen presenting cells, and express the inserted gene product (i.e. foreign gene product) in a manner that has the potential to be processed in the context of class I and/or class II MHC molecules; and (c) use as a recombinant vaccine in the treatment of certain tumors (Kantor, J. et al. (1992)).

A variety of other pox viruses have been useful as vectors for vaccines and the expression of foreign genes in cells, such as viruses of the genus *Avipoxvirus* such as, e.g., fowl pox, canary pox and viruses of the genus suipox such as swine pox.

It would be desirable to develop a vaccine which can induce immunological tolerance to nerve sheath proteins, particularly myelin proteins such as, e.g., MBP and MBP-related proteins.

SUMMARY OF THE INVENTION

We have discovered that by using a recombinant pox virus to intracellularly express a myelin protein or a T-cell eliciting epitope thereof, we can ameliorate or delay the symptoms of an inflammatory demyelinating disease in a vertebrate host. In particular, we have found that by intracellularly co-expressing the recombinant pox virus

and the myelin protein, the host immune system will thereafter substantially tolerate the myelin protein, thereby helping to ameliorate or delay the symptoms of the disease. By intracelluler expression, we mean that the process of transcription and translation of a protein occurs within the host cell. The protein, itself, may then be maintained in the cytoplasm, or transported to the nucleus, the cell membrane, other areas within the cell or into the extracellular space.

The recombinant pox virus has at least one insertion site containing a DNA segment encoding the myelin protein or a T-cell eliciting epitope thereof, which DNA segment is operably linked to a promoter capable of expression in the host. By the term "myelin protein" is meant those proteins which constitute the concentric oligodendrocyte wrapping around axons such as, e.g., MBP, PLP, MOP and MAG. A preferred myelin protein is MBP, MBP-related proteins, and T-cell eliciting epitopes thereof. Other proteins include MOP and MDP (myelodendritic protein).

In general, a preferred method of the present invention comprises introducing a sufficient amount of a first recombinant pox virus vector into a host to stimulate an immune response, and present the myelin protein to the immune systems. In some instances, one would want to contact the host with additional antigen at least once thereafter as a "boost". Preferably, one uses a first recombinant pox virus derived from a pox virus of a different genus for the "boost", e.g., first administer using a recombinant pox virus derived from an avipox, then boost with another derived from suipox. The first recombinant pox virus vector comprises a DNA segment which

encodes the myelin protein or T-cell eliciting portion thereof. The additional antigen as aforesaid may be added, e.g., by using a second recombinant pox virus from an immunologically distinct pox virus, typically from a different genus than the first pox virus.

Alternatively, or in conjunction with expression of myelin protein by the first recombinant pox virus, additional myelin protein or T-cell eliciting fragment thereof, may be added by contacting the host with the myelin protein formulated with an adjuvant or in a liposomal formulation or contacting the host with DNA encoding the myelin protein either as direct DNA or in an alternative viral vector.

The present invention also features a cell, preferably a homogeneous population of cells, which includes either the recombinant pox virus DNA or the recombinant pox virus, where the encoded myelin protein is expressed intracellularly in a sufficient amount to elicit an immune response against the myelin protein. The cell is preferably one which is capable of supporting virus or protein expression for at least 48 hours, such as, e.g., a mammalian, avian, reptilian, or insect cell line.

In another aspect, the present invention also features therapeutic compositions which include one or more recombinant pox virus disclosed herein where the virus(es) is provided in a pharmaceutically acceptable carrier. The therapeutic compositions can be administered to a mammal, preferably a human, as a means for ameliorating or delaying the onset of an inflammatory demyelinating disease such as, e.g., MS. The therapeutic compositions of the invention can be

administered alone or as adjuncts to known therapies for treating inflammatory demyelinating disease such as, e.g, Beta-interferon, adrenocorticotropic hormone (ACTH) and corticosteroids for the treatment of MS. Accordingly, the treatment of inflammatory demyelinating disease in, for example, mammals and in a domesticated animal such as, e.g., a dog, cat, horse, rabbit, mouse, pig, cattle, reptile, gerbil, bird, sheep, goat and the like, or a captive animal such as a primate (e.g., chimpanzees, etc.) is within the scope of the present invention.

The term " inflammatory demyelinating disease" means any immunological response which destroys, damages or removes the myelin sheath of nerve fibers. An inflammatory demyelinating disease in a mammal, particularly a human, can be identified by such damage.

The invention also features a method for generating an immune response to a myelin protein in a host, the method including:

a. contacting the host with a sufficient amount of the myelin protein or T-cell eliciting epitope thereof; and

b. at least one periodic interval thereafter, contacting the host with additional myelin protein or T-cell eliciting epitope thereof.

The invention also features a method of manufacturing a myelin protein capable of eliciting an immune response. The method includes the steps of:

a) synthesizing a recombinant pox virus, preferably an avipox such as fowl pox, or canary pox, a capripox, or a suipox such as swine pox, which virus includes an operably linked DNA segment encoding a

myelin protein, preferably MBP, MBP-related protein, or a T-cell eliciting epitope thereof;

b) introducing the recombinant pox virus into a mammalian host, preferably a human, under conditions which permit the infection of cells which support gene expression by the virus, preferably dermal cells or muscle cells; and

c) expressing and appropriately presenting the myelin protein intracellularly so that an immune response is elicited in the host.

The method is also useful for producing cells (or cell lysate thereof) which can be used to detect the onset of or evaluate the progression of an inflammatory demyelinating disease in a host. The cells appropriately present the myelin protein and are therefore recognized by T-cells isolated from the host. The killing by the T-cells can be detected by standard techniques such as, e.g., chromium release from cells labelled with radioactive chromium.

In another embodiment of the present invention, the method further comprises isolating the cells infected by the recombinant pox virus and using the cells, either as whole cells or cell fractions, as an immunogen for the production of antibodies, preferably monoclonal antibodies, which bind intracellularly expressed myelin protein in the infected cell. Of course, the antibody may also bind a T-cell eliciting epitope of the myelin protein. In accordance with conventional immunological techniques, such antibodies can readily be made by those skilled in the art.

In yet another embodiment of the present invention, the method

further comprises isolating cells from the infected host (or isolating the infected cells themselves), and using them, either as whole cells or cell fractions, to induce or stimulate an immune response in a second host to ameliorate or delay the onset of an inflammatory demyelinating disease in a mammal such as, e.g., a human.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 A and B shows two graphs which depict the clinical course of EAE in vAbT249 (control) and vT15 (MBP) vaccinated *C. jacchus* marmosets.

Figures 2 A, B, C and D shows four graphs which illustrate the proliferative responses in PBMC of WM-immunized *C. jacchus* marmosets vaccinated with vT15 (MBP) (Figures 2 A and C) and vAbT249 (control) (Figures 2B and D). Figures 2A and B show in vitro stimulation with MBP (50μg/ml). Figures 2C and D show stimulation with NYCBH vaccinia antigen (2μg/ml).

Detailed Description of the Invention

Viruses of the family *Poxviridae* are well known cytoplasmic viruses. Thus, genetic material expressed by recombinant pox viruses of the invention typically remains in the cytoplasm and does not have the potential for inadvertently integrating into host cell genes. Furthermore, because pox viruses have large genomes, they can readily be used to deliver a wide range of genetic material including multiple foreign genes (i.e., act as a multivalent vector).

Pox viruses disclosed herein are typically viruses of the family *Poxviridae*, preferably selected from the group consisting of avipox, capripox, orthopox, entomopox and suipox. Preferred avipox viruses include fowl pox, canary pox, pigeon pox, turkey pox, quail pox. An especially preferred avipox is fowl pox. Preferred orthopox viruses include vaccinia, cowpox, mousepox (ectromelia), rabbitpox, racoon pox, and monkey pox. An especially preferred orthopox is vaccinia. Preferably the suipox is swine pox, and the capripox is sheep or goat pox virus.

Where the host is a human, it is sometimes preferable to use a pox virus whose host range is restricted and does not include humans. Such a restriction can be natural such as by the use of avipox, capripox, entomopox or suipox in a human. Restriction can also be accomplished by attenuating (i.e. weakening) the pox virus whose host range is a human to a sufficient degree that pox virus will no be virulent. See for example, the NYVAC strain of vaccinia, U.S. Patent No. 5,494,807, herein incorporated by reference. See also the MVA strain in U.S. Pat. No. 5,185,146, herein incorporated by reference. The use of such attenuated pox viruses as vectors is particularly preferred in a host whose immune system is impaired.

Although less preferred than the pox viruses, other recombinant viruses (and DNA derived therefrom) are within the scope of the present invention. These viruses include those derived from herpes virus, polyoma viruses (e.g, SV40 and polyoma), adenoviruses, adeno associated viruses, as well as RNA viruses such as retroviruses and picornavirus such as polio virus, sindbis, Venezuelan equine

encephalitis. Other viruses include iridoviruses such as frog virus, and African swine fever virus.

One needs to select a parental virus that is not virulent for the putative host animal. For example, one can use a more attenuated virus, a non-replicating virus for that host, etc.

In preferred embodiments of the present invention, the recombinant pox virus exhibits a low replicative efficiency in the infected (i.e. target) cells of the host. Generally, replicative efficiency can be determined by conventional virological and cell culture techniques such as, e.g., infecting with virus and then quantitating the amount of viral progeny produced by the infected cell by titration on permissive cells (e.g. by viral plaque assay). The amount of progeny virus so produced can be expressed over the total number of cells used in the experiment. Preferred pox viruses used in accordance with the present invention preferably produce, on average, no more than about 10 productive (i.e., infectious) progeny per cell, more preferably, no more than about 1 productive progeny, still more preferably, no more than 0.1 progeny per cell. A productive progeny is one that will infect and replicate in a permissive host cell.

As a result of the low replication efficiency and the non-integrative, cytoplasmic nature of preferred pox viruses, the recombinant pox viruses made therefrom will not result in sustained replication and infection of other cells. Thus, the vector and transformed cells will not adversely affect cells in the host animal at locations distant from where the target cell is. Accordingly, more

virulent pox virus can be used to make the recombinant pox viruses of the present invention, provided that the virulence of the virus has been reduced by, e.g., selection or chemical or genetic mutagenesis so as to produce an attenuated strain of the virulent pox virus. See previously incorporated U.S. Pat. No. 5,494,807.

Although not wishing to be bound by theory we believe that the recombinant pox viral vector elicits a CD8+ response. The myelin encoded antigen specifically triggers the down regulation of myelin antigen-specific CD4 cells, perhaps through a CD8+ cell mediated response. Alternatively, there could be secretion of inhibitory cytokines by regulatory T-cells, or a combination of both.

Preparation of recombinant pox virus and vectors

Basic techniques for preparing recombinant pox viruses containing a heterologous DNA sequence encoding a foreign protein are well known in the art. For example, one method of preparing recombinant pox virus involves homologous recombination between the viral DNA sequences flanking the DNA sequence in a donor plasmid and homologous sequences present in the parental virus (Mackett, et al., *Proc. Natl. Acad. Sci. USA* 79:7415-7419 (1982)). See also methods for manufacturing recombinant fowlpox virus described in U.S. Patent No. 5,093,258, the disclosure of which is incorporated herein by reference.

Other techniques for making recombinant pox virus include using a restriction endonuclease site that is naturally present or artificially

inserted in the parental viral vector to insert the heterologous DNA. See U.S. Pat. No. 5,445,953, incorporated herein by reference.

Conventional recombinant DNA manipulations such as restriction enzyme digestion, ligation, transfection, transformation, agarose and polyacrylamide gel electrophoresis, DNA sequencing, electroporation are well known in the art and have been described (see e.g., Sambrook et al. in *Molecular Cloning: A Laboratory Approach* (1988); Ausubel et al. *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley Intersciences (1993)).

As an illustrative example, the heterologous DNA sequence to be inserted into a pox virus can be placed into a donor plasmid, bacteriophage or virus such as, e.g., an *E. coli* plasmid such as pBR322 or pUC19. In instances where a donor plasmid is chosen, the plasmid preferably includes an origin of replication (ORI), and one or more detectable markers such as, e.g., an antibiotic resistance gene (e.g., ampicillin or tetracycline) for propagation in *E. coli*. As is more fully described, infra, the heterologous DNA is typically flanked by DNA sequences which are homologous to the section of pox virus DNA flanking the insertion site.

Separately, and in addition, the heterologous DNA gene sequence to be inserted is ligated to a suitable promoter. The promoter-gene linkage is then operably positioned in the plasmid so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of pox DNA which is the desired insertion region. With a parental pox viral vector, a pox

promoter is generally used. The resulting recombinant plasmid is then used to transform *E. coli* where the cells are then propagated under selective conditions to reproduce the recombinant plasmid. The reproduced plasmid is then purified by standard means. Of course, other plasmids as well as a eukaryotic vector (e.g., SV40 propagated in green monkey cells) may be employed to propagate the heterologous DNA (See Sambrook et al. supra).

A preferred method of making a recombinant pox virus of the present invention is by inserting the heterologous DNA of the plasmid into a pox virus by genetic recombination. For example, the recombinant plasmid containing the heterologous DNA sequence to be inserted is transfected into a cell culture, e.g., chick embryo fibroblasts, along with the pox virus, e.g., fowl pox or swine pox. Recombination between homologous pox DNA in the plasmid and the viral genome respectively, results in a recombinant poxvirus modified by the presence of the promoter-gene construct in its genome. Preferably, the site of recombination is one which does not affect virus viability.

As noted above, the gene is inserted into a region (insertion region or site) in the virus which does not substantially affect virus viability of the resultant recombinant virus. By the term "virus viability" is meant the capability of the recombinant pox virus to produce infectious viral particles within about 48 hours in a permissive host cell. Of course, in some situations it may be desirable for the insertion to affect virus viability such as when the insertion attenuates the resulting recombinant pox virus. See discussion, *infra*.

If it is not desirable to substantially affect virus viability, the artisan can readily identify such regions by, for example, by testing pre-determined segments of virus DNA for regions that support genetic recombination without seriously affecting virus viability. One region that can readily be used and is present in many viruses is the thymidine kinase (TK) gene, a gene that has been found in virtually all pox virus genomes examined [leporipoxvirus: Upton, et al.(1986) (shope fibroma virus); capripoxvirus: Gershon, et al.(1989) (Kenya sheep-1); orthopoxvirus: Weir, et al.(1983) (vaccinia); Esposito, et al. (1984) (monkeypox and variola virus); Hruby, et al.(1983) (vaccinia); Kilpatrick, et al. (1985) (Yaba monkey tumor virus); avipoxvirus: Binns, et al. (1988) (fowlpox); Boyle, et al. (1987) (fowlpox); Schnitzlein, et al. (1988) (fowlpox, quailpox); entomopox (Lytvyn, et al., (1992).

In vaccinia, in addition to the TK region, other insertion regions which do not substantially affect virus viability include, for example, the HindIII M fragment.

In fowlpox, in addition to the TK region, other insertion regions include, for example, the BamHI J fragment [Jenkins, et al., (1991)] the *Eco*RI-*Hind*III fragment, *Eco*RV-*Hind*III fragment, *Bam*HI fragment and the *Hind*III fragment set forth in EPO Application No. 0 308 220 A1. [Calvert, et al., (1993); Taylor, (1988); Spehner, et al., (1990) and Boursnell, et al. (1990)].

In swinepox preferred insertion sites include the thymidine kinase gene region.

In addition to the requirement that the heterologous DNA be inserted into an insertion region, successful expression of the inserted DNA by the recombinant pox virus requires the presence of a promoter operably linked to the heterologous DNA, i.e., the heterologous DNA, promoter and pox virus are in a suitable spatial relationship which supports transcription of the heterologous DNA. The promoter must be placed so that it is located upstream from the DNA to be expressed. Promoters are well known in the art and can be readily selected depending on the host and the cell type desired. For example in pox viruses, promoters derived from pox viral promoters are used. They can be based upon native promoters such as the vaccinia 7.5K or 40K or fowlpox promoters such as FPV C1 or they can be artificial constructs containing appropriate pox sequences. Enhancer elements can also be used in combination with promoters to increase the level of expression, although enhancers need not always be located upstream of the inserted heterologous DNA. Furthermore, the use of inducible promoters such as, e.g., heat or metal inducible promoters, are also well known in the art and will be preferred in some instances.

It is not necessary that the pox vector encode all pox proteins. Rather, one needs only so much of a pox virus as is necessary for infecting the target cell and to permit expression of the heterologeous protein. This is sometimes referred to as corresponding to a sufficient portion of the pox genome for infection and expression.

A recombinant pox virus of the invention may optionally include a marker, such as β-galactosidase, CAT, neomycin or methotrexate resistance, whereby the target cells of the host may be detected (or

selected). The use of such a marker allows the skilled artisan to screen various viral vectors for those that are non-lytic or non-cytopathic in a particular target host cell. For example, the gene encoding β-galactosidase (*lac*Z) can be inserted into any recombinant pox virus disclosed herein, whereby the modified virus vector is then introduced into the target host cell and the production of β-galactosidase is measured. Expression of β-gal provides an indication of viral infectivity and gene expression.

IMMUNE RESPONSES

A specific immune response against a myelin protein or T-cell eliciting epitope thereof, can be generated by administering between about 10^5-10^9 pfu of a recombinant pox virus of the invention, to a host. More preferably about 10^7-10^9 pfu is used, although this amount may vary depending several factors such as, e.g., the particular host used. The preferred host is a mammal such as, e.g., a domesticated animal, a captive animal, or a human. In some instances, it is desirable to "boost" the presentation by administering additional antigen to the host. This may be one to three months later. There may also be at least a second "boost" preferably one to three months after the first boost. The myelin protein T-cell eliciting epitope thereof may be administered using the same pox virus vector or, more preferably the antigen is administered using a second pox virus vector from an antigenically unrelated pox virus, or alternatively, the antigen may be administered directly using, for example, an adjuvant or liposome in a pharmaceutically acceptable carrier. Cytokines, e.g., IL-2, IL-6, IL-12 may be used as biologic adjuvants and can be administered systemically to the host. Alternatively cytokines or co-stimulatory

molecules, e.g., B7.1, B7.2, can be co-administered via co-insertion of the genes encoding the molecules into the recombinant pox vector.

Adjuvants include, for example, RIBI Detox (Ribi Immunochemical), QS21 and incomplete Freund's adjuvant. Methods for making liposomes for administration are known.

T-Cells

T-cells that react against the epitope(s) of myelin protein or a T-cell eliciting epitope thereof can be obtained from peripheral blood mononuclear cells (PBMC) by standard methods. For example, PBMC can be separated by using Lymphocyte Separation Medium gradient (Organon Teknika, Durham, NC, USA) as previously described [Boyum, et al., *Scand J. Clin Lab Invest* 21: 77-80 (1968)]. Washed PBMC are resuspended in a complete medium, for example, RPMI 1640 (GIBCO) supplemented with 10% pool human AB serum (Pel-Freeze Clinical System, Brown Dear, WI, USA), 2mM glutamine, 100 U/ml penicillin and 100 μg/ml of streptomycin (GIBCO). PBMC at a concentration of about 2 x 10^5 cells in complete medium in a volume of, for example, 100 μl are added into each well of a 96-well flat-bottom assay plate (Costar, Cambridge, MA, USA). Protein antigen such as selected MBP peptides is then added into the cultures in a final concentration of about 50 μg/ml and incubated at 37°c in a humidified atmosphere containing 5% CO_2 for 5 days. After removal of the MBP or cytotoxic T-cell eliciting epitope thereof from the media, the cultures are provided with fresh human IL-2 (10U/ml) after 5 days and replenished with IL-2 containing medium every 3 days. Primary cultures are restimulated with the same peptide (50 μg/ml) on day 16. 5 x 10^5 irradiated (4,000

rad) autologous PBMC are added in a volume of about 50 μl complete medium as antigen-presenting cells (APC). About five days later, the cultures are provided with human IL-2 containing medium as described previously. Cells are restimulated for 5 days at intervals of 16 days.

Epitope mapping

T-cells prepared as described herein can be used to identify the epitope(s) of a myelin protein or fragment thereof, that are recognized by T-cells including cytotoxic T-cells. One method of preparing a T-cell eliciting epitope of a myelin protein includes limited proteolytic digestion of the protein with enzymes such as, e.g., papain, trypsin, or chymotrypsin (supplied by SIGMA Chemical Co. St. Louis, Mo.). Alternatively, fragments of a myelin protein can be chemically synthesized by conventional methods such as, e.g., the Merrifield Solid-Phase Technique. For example, cytotoxic T-cells can then be plated and the different myelin protein fragments added to different wells. Only cytotoxic T-cells that specifically recognize (i.e., bind) a peptide fragment with at least one epitope will continue to expand, thereby permitting ready identification.

T-cell eliciting epitopes of a myelin protein can be used as an alternative to using the entire protein. Additionally, one can prepare other fragments containing the epitope to enhance its ability to elicit a T-cell response.

Variants of Myelin Protein and DNA

Variants of myelin protein and DNA are within the spirit and scope of the present invention. For example, a degenerate variant of

the MBP DNA sequence is the same as that MBP sequence, except that the degenerate variant includes at least one nucleotide change which results in one or more alternative codons being used to encode the MBP amino acid sequence. The artisan will appreciate that there are 61 codons for the 20 common amino acids so that many of the amino acids are encoded by more than one (alternative) codon (see Darnell et al. eds., Scientific American Books, Inc., 1986).

Additional variants are within the spirit and scope of the present invention. For example, a recombinant pox virus of the invention may include the MBP DNA sequence, except that the variant will include at least one nucleotide change which results in one or more alternative codons being used to encode the MBP amino acid sequence, whereby the alternative codon encodes a conservative amino acid, i.e. an amino acid which can substitute for another amino acid because of similar characteristics. Examples of conservative amino acid substitutions include, e.g., valine for glycine, arginine for lysine, leucine for valine, serine for threonine, etc. Of course, a conservative amino acid substitution, by definition, will not substantially affect T-cell eliciting epitope(s) of MBP.

In yet another example of a variant, a recombinant pox virus of the invention may include the MBP DNA sequence, except that the variant will include at least one nucleotide change which results in one or more alternative codons being used to encode the MBP amino acid sequence, whereby the alternative codon encodes a non-conservative amino acid, i.e., an amino acid with different characteristics than the corresponding amino acid in the MBP amino acid sequence. In this

example, the non-conservative amino acid substitution does not substantially affect T-cell eliciting epitope(s) of MBP.

Alternatively, or in addition, the recombinant pox virus may include deletions of the MBP DNA sequence, or insertion of foreign DNA into the MBP DNA sequence, provided that the deletions and/or insertions do not materially affect a T-cell eliciting epitope(s) of MBP.

A T-cell eliciting epitope of MBP in a polypeptide fragment is another type of MBP variant.

Therapeutic Compositions

The recombinant pox viruses of the present invention can be administered to a suitable host using any acceptable route, including, for example, scarification and injection, e.g., intradermal, subcutaneous, intramuscular, intravenous or intraperitoneal.

A therapeutic composition of the present invention will suitably include one or more recombinant pox virus of the invention, which composition will typically be administered in unit dosage form to a suitable host in a sterile aqueous or non-aqueous solution, suspension or emulsion in association with a pharmaceutically-acceptable carrier such as physiological saline. Formulations for parenteral administration may also contain as common excipients polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated napthalenes and the like. (See generally *Remington's Pharmaceutical Sciences* (Mack Pub. Co., Easton, PA, 1980).

The therapeutic compositions of the invention may be employed as the sole active agent in a pharmaceutical or can be used in combination with other compounds and/or therapies which serve to treat inflammatory demyelinating disease.

The amount of the recombinant pox virus in the therapeutic composition will vary depending on a number of factors, including the dosage of the virus administered, the virulence of the selected virus, and the route of administration. In general terms, a composition is provided in aqueous physiological buffer solution containing about 10^5 to 10^9 pfu for parenteral administration. When the host is a human, typical dose ranges will include about 10^5 to 10^9 pfu recombinant avipox or suipox virus, preferably fowl pox or swine pox. However, the preferred dosage of the therapeutic composition is likely to depend on such variables as the type and extent of progression of the inflammatory demyelinating disease, the overall health status of the patient, the relative virulence and biological efficacy of the particular recombinant pox virus selected, the formulation of the composition excipients and its route of administration.

REFERENCE EXAMPLE 1
CONSTRUCTION OF VECTORS

Pox Viruses

A number of pox viruses have been developed as live viral vectors for the expression of heterologous proteins (Cepko et al., (1984); Morin et al., (1987); Lowe et al., (1987); Panicali & Paoletti, (1982); Mackett et al., (1982)). Representative fowlpox, swinepox virus and vaccinia are available through the ATCC under accession numbers VR-229, VR-363, and VR-325 respectively.

DNA Vectors For *In Vivo* Recombination With A Parent Virus

Genes that code for MBP or a T-cell eliciting epitope thereof, are inserted into the genome of a pox virus such as e.g., fowl pox virus, swine pox or vaccinia in such a manner as to allow them to be expressed by that virus along with the expression of the normal complement of parent virus proteins. This can be accomplished by first constructing a DNA donor plasmid for *in vivo* recombination with a pox virus.

In general, the DNA donor plasmid contains the following elements:

(i) a prokaryotic origin of replication, so that the vector may be amplified in a prokaryotic host;

(ii) a gene encoding a marker which allows selection of prokaryotic host cells that contain the vector (e.g., a gene encoding antibiotic resistance);

(iii) at least one gene encoding a desired myelin protein (e.g., MBP), each located adjacent to a promoter capable of directing the expression of the gene; and

(iv) DNA sequences homologous to the region of the parent virus genome where the foreign gene(s) will be inserted, flanking the construct of element (iii).

Methods for constructing donor plasmids for the introduction of multiple foreign genes into pox virus are described in W091/19803, the techniques of which are incorporated herein by reference. In general, all DNA fragments for construction of the donor vector, including fragments containing transcriptional promoters and fragments containing sequences homologous to the region of the parent virus genome into which foreign genes are to be inserted, can be obtained from genomic DNA or cloned DNA fragments. The donor plasmids can be mono-, di-, or multivalent (i.e., can contain one or more inserted foreign gene sequences).

The donor plasmid preferably contains an additional gene which encodes a marker which will allow identification of recombinant viruses containing inserted foreign DNA. Several types of marker genes can be used to permit the identification and isolation of recombinant viruses. These include genes that encode antibiotic or chemical resistance (e.g., see Spyropoulos et al.(1988); Falkner and Moss.(1988); Franke et al.(1985), as well as genes such as the *E. coli lac*Z gene, that permit identification of recombinant viral plaques by colorimetric assay (Panicali et al.(1986)).

Integration Of Heterologous DNA Into Pox Virus

Homologous recombination between donor plasmid DNA and viral DNA in an infected cell results in the formation of recombinant viruses that incorporate the desired elements. Appropriate host cells for *in vivo* recombination are generally eukaryotic cells that can be infected by the virus and transfected by the plasmid vector. Examples of such cells suitable for use with a pox virus are chick embryo fibroblasts, HuTK143 (human) cells, and CV-1 and BSC-40 (both monkey kidney) cells. Infection of cells with pox virus and transfection of these cells with plasmid vectors is accomplished by techniques standard in the art (Panicali and Paoletti, U.S. Patent No. 4,603,112, WO89/03429, both references herein incorporated by reference).

Following *in vivo* recombination, recombinant viral progeny can be identified by one of several techniques. For example, if the DNA donor vector is designed to insert one or more foreign genes into the parent virus thymidine kinase (TK) gene, viruses containing inserted DNA will be TK^- and can be selected on this basis (Mackett et al., (1982)). Alternatively, co-integration of a gene encoding a marker or indicator gene with the foreign gene(s) of interest, as described above, can be used to identify recombinant progeny. One preferred indicator gene is the *E. coli lacZ* gene: recombinant viruses expressing β-galactosidase can be selected using a chromogenic substrate for the enzyme (Panicali et al.(1986)).

Characterizing Antigens Expressed By Recombinant Pox Viruses

Once a recombinant pox virus has been identified, a variety of methods can be used to assay the expression of the polypeptide

encoded by the inserted gene. These methods include black plaque assay (an *in situ* enzyme immunoassay performed on viral plaques), Western blot analysis, radioimmunoprecipitation (RIPA), and enzyme immunoassay (EIA). Antibodies that recognize a myelin protein such as, e.g., MBP, MBP-related proteins, or T-cell eliciting epitopes thereof, are commercially available or, alternatively, can be made by conventional techniques. For example, monoclonal antibodies against these MBP, MBP-related proteins, or T-cell eliciting epitopes thereof, can be made by employing standard hybridoma technology (see, e.g., Kohler et al. (1975); Kohler et al. (1976); Hammerling et al. In *Monoclonal Antibodies and T Cell Hybridomas*, Elsevier NY, 1981; Harlow and Lane in *Antibodies: A Laboratory Approach* CSHSQB (1988); and Ausubel et al. supra).

Measurement of Antibody Titers

Prior to each immunization and 2 weeks following each immunization, anti-MBP antibody was quantified by ELISA. For example, microliter plates were coated with MBP (100 ng/well, SOURCE), ovalbumin (100 ng/well, Sigma), or $1x10^7$ PFU/well UV-inactivated V-Wyeth in PBS. The plates were blocked with 2% BSA in PBS, dried, and stored at -20° C until used. The plates were incubated with serum diluted 1:5, as well as a monoclonal antibody for PSA (DAKO M750, Denmark) as a standard control, for 24 hours at 4° C. Plates were washed several times with PBS containing 1% BSA, and incubated at 37° C for 45 min with horseradish peroxidase-conjugated goat anti-human IgG or IgM heavy chain specific antiserum (1:8000) (Southern Biotechnology Associates, Birmingham, AL) and antibody detected by HRP substrate system (Kirkegaard & Perry Laboratories,

Gaithersburg, MD) according to the manufacture's instructions. The absorbance of each well was read at 405 nm using a Bio-Tek EL310 microplate ELISA reader (Winooski, VT).

Optimizing immune responses to recombinant pox virus

Administration of a recombinant pox virus disclosed herein may, in some settings, elicit an undesirably strong immune response to the pox virus vector. Repeated administration of the same vector in subsequent boosts might result in undersirably rapid clearance of the virus from the host, not allowing sufficient time to express the myelin protein and boost the immune response to it. Thus while numerous "boosts" with recombinant pox virus of the invention are possible, repeated use of any one virus thereof may not always be preferred. The use of recombinant pox viruses from an antigenically distinct pox virus can in most cases minimize this problem.

In accordance with the present invention, to minimize high level immune response to a recombinant pox virus, e.g., when the first pox virus (or group of recombinant pox viruses) is fowl pox, the second and subsequent recombinant pox virus is selected from a different genus such as an orthopox, e.g. vaccinia, or a suipox.

Alternatively, another method of minimizing an undesirable immune response is to select an appropriate pox virus which exhibits a suitable host range and/or tissue specificity. For example, pox virus can be selected whose primary host range is different than the animal that the gene delivery system is to be used in. For example, avipox such as, e.g., fowl pox, or suipox such as, e.g, swine pox can be

used when the host is a non-permissive mammal such as a human. As an example, for those veterinary applications where the host is a pig, the use of suipox would not be advantageous. Accordingly, attenuated strains of pox viruses may be desirable when another pox virus outside the host range of the host is not available. For example, highly attenuated strains of certain orthopox viruses such as vaccinia (MVA strain) may be used or alternatively, may be further modified by conventional genetic or chemical mutagenesis techniques to be even more attenuated or non-virulent in the normal host range.

The cell specificity of the pox virus of interest is one way to easily screen for infectivity and replication efficiency.

EXAMPLE 1

Construction of recombinant fowlpox virus expressing MBP

The gene encoding MBP was excised from a 2.2 kb cDNA fragment isolated from a human brain cDNA library [Kamholz *et al.*, PNAS (USA) 83:4962-4966 (1986)]. A 1.2 kb DNA fragment, containing the entire 516 nucleotide coding sequence for MBP, 36 nucleotides of the 5' untranslated region, and 661 nucleotides of the 3' untranslated region, was inserted into a fowlpox virus plasmid vector. The resulting plasmid, designated pt3064, is used to make vT92, which contains the MBP gene under the control of the vaccinia virus 40K early/late promoter (Gritz *et al.*, 1990) and the *E. coli lacZ* gene under the control of the fowlpox virus C1 promoter (Jenkins *et al.*, 1991). This plasmid was deposited with the ATCC on March 22, 1996 and given accession number 97491. The foreign sequences are flanked by DNA sequences from the Bam HI J region of the fowlpox

virus genome (Jenkins *et al.*, 1991). A derivative of the POXVAC-TC (Schering Corp.) vaccine strain of fowl pox virus was used as the parental virus. The generation of recombinant fowlpox virus was accomplished via homologous recombination between fowlpox sequences in the parental fowlpox virus genome and the corresponding sequences in pT3064 in fowlpox-infected chick embryo dermal (CED) cells transfected with pT3064. Recombinant virus, designated vT92, was identified and plaque-purified by growth on CED cells in the presence of Bluogal (Life Technologies; Gaithersburg, MD), a chromogenic substrate for β-galactosidase. Insertion of the MBP gene into the fowlpox genome was confirmed by amplification of the inserted sequences by polymerase chain reaction (PCR). Expression of MBP was demonstrated by Western analysis using MBP-specific antisera.

Construction of recombinant vaccinia virus expressing MBP

The gene encoding for human MBP was derived from a 2.2 kb cDNA fragment isolated from a human cDNA library [21]. A 1.2 kb DNA fragment containing the 516 coding nucleotide sequence for MBP, 36 nucleotide of the 5' untranslated region, and 661 nucleotides of the 3' untranslated region, was inserted into a vaccinia virus transfer vector. The resulting plasmid, designated pT115, containing the MBP gene under the control of the vaccinia virus early/late promoter [22] flanked by DNA sequences from the Hind III M region of the vaccinia genome. These flanking sequences include the vaccinia K1L host range gene which is required for multiplication in human cells [23]. The plasmid was deposited with the ATCC on March 22, 1996 and given accession number 97490. A derivative of the New York City

Health (NYCBH) strain of vaccinia was used as the parental virus in the construction of recombinant vaccinia virus. This parental virus was designated vAbT33 (parent). This was deposited with the ATCC on May 15, 1989 and given ATCC No. VR-2240. The parental virus lacks a functional K1L gene and thus cannot efficiently replicate on rabbit kidney RK13 cells [24].

The generation of recombinant vaccinia virus was accomplished via previously described standard homologous recombination techniques. Briefly, homologous recombination was performed between vaccinia virus sequences in the vAbT33 (parent) genome and the corresponding sequences in RK13 cells infected with the parental virus and transfected with the pT115 vector. The RK13 cell line is publicly available (ATCC: Accession No. CCL37). A recombinant vaccinia virus bearing the MBP gene was selected by growth on RK13 cells. One recombinant vaccinia virus isolate included the entire MBP gene by Southern blot hybridization of restriction enzyme digests. The recombinant virus was designated vT15 (MBP). **Animals**

Marmosets (*C. jacchus)* were maintained in primate colonies at the University of California, San Francisco (UCSF). The animals used in this study were cared for in accordance with the guidelines of the Committee on Animal Research at UCSF and those of the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. A maximum of 2.5 ml of blood every other week was taken from each animal. Phlebotomy and immunization were done under brief anesthesia (ketamine, 10 mg intramuscularly).

Western Analysis of MBP Protein Expression

Expression of MBP was demonstrated in vT15(MBP) infected cell lysates by Western blot analysis using the polyclonal rabbit anti-guinea pig MBP R 120. This polyclonal antibody binds human MBP. The preparation of the antibody has been previously described ([25]. A control recombinant vaccinia virus was prepared which includes the gene encoding the equine herpes virus gH was prepared using homologous recombination as described above. The resulting recombinant vaccinia virus was designated vAbT249 (control).

Briefly, western experiments were conducted by infecting BSC-40 cells with either parental vaccinia virus (designated vAbT33) or the recombinant vaccinia virus (vT15 (MBP) in Dulbecco's Modified Eagle's Medium containing 2% fetal bovine serum. The infected cells were lysed in hypotonic lysis buffer (150 mM NaCl, 0.05% EDTA, 10 mM KCl, 1 mM PMSF) and then sonicated. Cell lysates and culture media were electrophoresed on an SDS-10% acrylamide gel. The proteins were transblotted to nitrocellulose, and the blot was incubated with a antibody specific for MBP (R120) at ambient temperature, washed, and then incubated with goat anti-rabbit phosphatase-labeled secondary antibody (AP, Kirkegaard & Perry Laboratories, Gaithersburg, MD) and developed according to the manufacturers instructions.

Vaccination with recombinant vaccinia viruses

Eight marmosets were vaccinated with about 10^7 pfu of either vAbT249 (control) or vT15 (MBP) in 4 subcutaneous injections in the back. In some animals, the initial injection was followed eight weeks later by a booster injection of 10^9 pfu. In a single monkey, we

observed generalized cowpox lesions and transient fever from 10-14 days after first vaccination. There were no other detectable side-effects over this time interval. The pathological and immunological consequences of vaccinating the animals were evaluated by measuring pathological manifestations such as, e.g., the appearance and severity of skin lesions, anti-vaccinia T-cell activity, and antibody responses as described fully below.

Induction of EAE in marmosets

21 to 35 days after vaccination with either vAbT249 (control) or vT15 (MBP), all animals were immunized with 100 mg human whole WM in complete Freund's adjuvant supplemented with 3 mg/ml H37Ra. On the day of immunization and again 48 hrs later, animals received intravenously 10^{10} killed *Bordetella pertussis* organisms (obtained from Lederle laboratories, NY). The onset and progression of EAE was monitored by daily clinical examination of the marmosets using standard pathologic criteria (see generally Massacesi, L., et al. (1995)). At the termination of experiments, animals were euthanized under ketamine anesthesia by exsanguination followed by a lethal dose of intravenous pentobarbital (20 mg/kg).

PBMC Infected with recombinant vaccinia virus intracellularly express MBP

Expression of MBP was monitored *in vitro* in Epstein-Barr virus-transformed B-lymphoblastic cell lines (B-LCL) infected with vT15 (MBP) or VAbT249 (control). MBP Expression was also monitored *in vivo* in PBMC every 2-3 weeks following vaccination. Cytospin preparations (Shandon) were fixed in 50% ethanol, blocked with 1%

bovine serum albumin in phosphate buffered saline and stained with R 120 rabbit polyclonal anti guinea pig MBP (1:200), after washing slides were incubated with peroxidase-conjugated anti rabbit IgG (Sigma). Color development was achieved using 3-amino-9-ethyl-carbazole (Sigma) and slides were counterstained with hematoxylin/eosin.

Briefly, expression of MBP in B-LCL cells was conducted in accordance with the following method: Monkey autologous B lymphoblastoid cell lines (BLCL) were established by infecting $1x10^5$ freshly isolated PBMCs in 100 ml of RPMI 1640 supplemented with L-glutamine, gentamicin, and 10% FCS (Biofluids, Rockville, MD) with 100 ml supernatant from S594 cells (kindly provided by Dr. M. D. Miller, Harvard Medical School, New England Regional Primate Research Center, Southborough, MA), which contains the baboon herpesvirus *Herpes papio*, in a 96 well, flat-bottomed plate (Costar, Cambridge, MA). Following transformation, cells were expanded, and media changed once weekly.

We found that recombinant vaccinia virus vT15 expressed the myelin protein in PBMC obtained from vaccinated animals. This was not seen with a control group of marmosets vaccinated with vAbT249 (control). In each animal, the period during which MBP was expressed varied between 15-45 days after the first or initial vaccination.

Immune responses against MBP and vaccinia antigens

T-cell responses were measured in a standard 72 hr. proliferation assay [13] using ^{3}[H]-thymidine incorporation in 10^5 freshly isolated PBMC plated in 96 well round bottom plates. Briefly, each plate contained in 200 μl/AIM V (Gibco-BRL) and one of the following: no

addition (control); MBP 50 μg/ml; Proteolipid protein (PLP) 10 μg/ml; PHA 2.5 μg/ml; WM 0.1% (wt/vol.); NYCBH vaccinia strain 2 μg/ml. Stimulation indices were calculated as the ratio of unstimulated to stimulated PBMC. The term "stimulation index" means the ratio of the amount of T-cell activity in PBMC that is detected in a biological sample exposed to a recombinant vaccinia virus of the invention to the amount of T-cell activity in PBMC not exposed to the virus. Animals were terminated by euthanasia; spleen and lymph nodes harvested and then tested in the T-cell proliferation assays.

The production of MBP and vaccinia virus binding antibodies were followed every 14-21 days using a dot-blot filtration apparatus according to the manufacturer's instructions (Biorad) [13]. Briefly, 250 ng MBP or 500 ng NYCBH strain were adsorbed on 0.45 μm nitrocellulose filter membranes (Biorad), blocked and incubated in succession with a) serial dilutions of marmoset sera; b) anti-monkey IgG conjugated to peroxidase (Sigma, 1:4,000 dilution); c) diaminobenzidine (Pierce) as substrate for color development.

The vT15 (MBP) recombinant vaccinia virus alleviates EAE

In control animals vaccinated with vAbT249 (control), EAE developed within about 14-18 days after immunization with WM (Table I and Figure 1A), in agreement with prior work [12, 13, 26]. One control animal did not develop clinical EAE but unexpectedly died from anesthesia at day 14 after immunization. Table I is shown below:

Table I. Day of onset of EAE and neuropathologic findings in C. *jacchus* marmosets vaccinated with vT15 and vAbT249 vaccinia virus and immunized with WM.

Animal number	Vaccination	Clinical signs (day pi)	Sacrifice (day pi)	Inflammation	Demyelination
94-92	vT15	97	156	+ +	+ +
91-92	(MBP)	37	73	+ + +	+ +
344-92	"	none	63	+	-
353-92	"	none	63	+	-
326-91	vAbT249	14	120	+ + +	+ + +
499-92	(control)	18	163	+ + +	+ +
346-92	"	17	22	+ + +	+ + +

Day pi: day after induction of EAE by active immunization with WM.

In animals vaccinated with the recombinant vaccinia virus expressing MBP (vT15 (MBP)), clinical manifestations of EAE were markedly delayed (Table I and Figure 1B). For example, in two animals (animal nos. 344-92 and 353-92) no clinical signs of EAE were noted over a period spanning 63 days.

Clinical and pathological EAE in the vT15-vaccinated animals were consistently delayed or suppressed compared to the vAbT249-vaccinated controls. In previous experience with immunization of over 30 animals with WM in adjuvant/*bordetella pertussis*, over 75% of monkeys developed disease within 14-28 days and all developed disease by day 60 after immunization. Thus, it is not likely that the delayed onset of EAE in the present study (37-120 days) were due solely to the individual susceptibility of the animals in the vT15(MBP)-vaccinated group. The data indicate that the protective effects of vT15 (MBP) vaccination were specifically mediated through expression of MBP and were not due to a nonspecific modulation of immune responses by exposure to vaccinia virus.

Neuropathological examination was performed on one vaccinated control animal (animal no. 346-92) at the time of acute EAE (22 days after immunization and 5 days after onset of EAE, see Figure 1A).. In addition, two animals vaccinated with vT15 (MBP) (animal nos. 344-92 and 353-92, see Figure 1B) were studied 63 days after the first immunization. In the control animal, we found pathology typical of acute EAE in *C. jacchus* [12, 13]. For example, in the CNS white matter we observed multiple areas of perivascular infiltration comprised of mononuclear cells and macrophages. These

manifestations were accompanied by prominent concentric demyelination and early gliosis. No involvement of gray matter structures was found. These pathological manifestations were identical to those previously reported for untreated *C. jacchus* immunized with WM in adjuvant, indicating that vaccination with the control vaccinia virus did not modify EAE. By contrast, in the two vT15-vaccinated animals studied at day 63 after immunization, scarce perivascular infiltration was found and was unaccompanied by demyelination (Figure 1, Table I). Accordingly, by both clinical and neuropathologic criteria, prior vaccination with recombinant MBP-vaccinia protected *C. jacchus* marmosets against EAE.

Despite being an FDA approved vaccine component, vaccinia virus in some instances can have encephalitogenic properties (Paoletti et al. (1993)). We therefore investigated whether vaccination with the NYCBH strain of vaccinia virus could by itself augment this EAE in *C. jacchus*.

Accordingly, two marmosets from each group vaccinated with the vAbT249 (control) or vT15 (MBP) recombinant vaccinia virus and then injected with white matter (WM) were followed for a period of 73-163 days after injection of the WM (see Table I and Figures 1A and B). In both groups, some animals developed severe clinical signs (grade 3 and above) resulting in death or requiring euthanasia (see animal nos. 91-92 (at 70 days); 94-92, 326-91 and 499-92 (each at 120 days)). In the control animals, disseminated, extensive infiltration of both white and gray matter structures accompanied by extensive demyelination were found (Table I). However, in two animals

vaccinated with vT15 (MBP), no such clinical manifestations were observed prior to sacrificing the animals (Figure 1B). These observations indicate that acute disseminated encephalomyelitis may follow some vaccinations with vaccinia virus. Reducing the dosage, using an even more attenuated strain of vaccinia or using an alternative pox vector such as an avipox or suipox are expected to avoid such occurrences.

Immune response against myelin and vaccinia antigens

1. T-cell responses

^{3}[H]-thymidine incorporation in response to stimulation with MBP, proteolipid protein (PLP) and vaccinia virus (NYCBH) was serially measured in PBMC beginning on the day of vaccination and until the time of death. The results are summarized in Figure 2.

T-cell reactivity against MBP: In two marmosets vaccinated with vT15 (MBP), transient and modest (i.e. stimulation index of 2) proliferative responses were observed prior to induction of EAE with WM (animal nos. 344-92 and 353-92, see Figures 2A and C). This result indicates that vaccination with the recombinant vaccinia virus presents the autoantigen to the immune system in vivo. Following active immunization with WM, T-cell proliferative responses against MBP were observed by days 15 and 22 in two of the vAbT249 (control) vaccinated animals and by day 62 in the third control monkey. However, T-cell proliferation against MBP did not develop in the vT15 (MAB) vaccinated monkeys up to 74 days after immunization. In one animal in this group (animal no: 91-92), T-cell responses against MBP

remained negative during the entire experiment. In no instance was T-cell reactivity against PLP detected in these experiments.

T-cell reactivity against vaccinia virus: Strong T-cell proliferative responses against NYCBH were observed in both the experimental and control groups following either vaccination or immunization with WM (see Figures 2C and D). This indicated that subcutaneous injection of the recombinant vaccinia viruses used herein efficiently stimulated cellular immune responses in *C. jacchus*. In animals later developing acute disseminated encephalomyelitis, anti-vaccinia T-cell responses increased.

T-cell reactivity against PLP: No animal exhibited T-cell reactivity against PLP in response to immunization with WM. However, one animal developing EAE exhibited a strong proliferative response against PLP.

Antibody responses

A) Antibodies against MBP: Serum antibody titers were not detected following vaccination with either the vT15 (MBP) or vAbT249 (control) recombinant vaccinia viruses. However, antibodies which bind MBP developed 14-21 days after immunization with WM in all animals.

b) Antibodies against vaccinia virus: Antibodies against the NYCBH strain of vaccinia virus were detectable 14-21 days following the first vaccination. This result indicates that, like T-cell responses, humoral immunity against vaccinia antigens had been induced by

vaccination.

Presentation of antigens by vaccinia virus vectors is achieved in part via the endogenous pathway of antigen presentation in association with MHC class I antigens. This could stimulate the proliferation of regulatory T-cells, in particular CD8+ T-cells. Without wishing to be bound to any particular theory, the alleviation of EAE observed in these experiments may be due to either immunological suppression of certain subsets of T-cells, or secretion of inhibitory cytokines by regulatory T-cells, or both. These protective mechanisms (or others) may be negated by the encephalitogenic properties of vaccinia virus.

Accordingly, to provide a recombinant pox virus with reduced encephalitogenic potential, the recombinant fowl pox virus disclosed above can be used in place of the recombinant vaccinia virus, in order to ameliorate or delay the onset of an inflammatory demyelinating disease in a mammal, particularly domesticated animals, captive animals, or humans.

It will be readily apparent that some of the above-described compositions and methods can be used as a kit suitable for clinical or veterinary use. Such a kit would include one or more recombinant pox virus of the invention in a pharmaceutically acceptable carrier, or cell or homogeneous population of cells which include the recombinant pox virus. In particular, the cells or homogeneous population of cells can be part of a diagnostic kit whereby the cells or homogeneous population of cells intracellularly express a myelin protein, preferably MBP, MBP-related protein, or a T-cell eliciting epitope thereof, which

cells or homogeneous population of cells are capable of binding T-cells derived from a host suspected of having or predicted to suffer from an inflammatory demyelinating disease as described herein. In accordance with conventional immunological technique, the binding between the T-cells and the cells or homogeneous cells disclosed herein can be detected.

All publications mentioned in the specification are indicative of the level of skill of those in the art to which this invention pertains. All publications are hereby incorporated by reference to the same extent as if each individual publication were specifically and individually stated to be incorporated by reference.

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Although the foregoing invention has been described in some detail by way of illustration and example for the purposes of clarity of understanding, one skilled in the art will easily ascertain that certain changes and modifications may be practiced without departing from the spirit and scope of the appended claims.

We claim:

1. A recombinant pox virus derived from a pox virus, where the recombinant pox virus has at least one insertion site containing an operably linked heterologous DNA sequence encoding a myelin protein, wherein the recombinant pox virus is capable of modulating a host immune response.

2. The recombinant pox virus of claim 1, wherein the heterologous DNA sequence encodes MBP or a variant thereof.

3. The recombinant pox virus of claim 1, wherein the pox virus is an orthopox, avipox or suipox virus.

4. The recombinant pox virus of claim 3, wherein the pox virus is selected from the group consisting of vaccinia, fowl pox, or swine pox.

5. The recombinant pox virus of claim 1, wherein the pox virus is wild-type or attenuated.

6. The recombinant pox virus of claim 1, wherein the immune response is a T-Cell response.

7. A cell infected with the pox virus of claim 1, wherein the cell expresses the myelin protein and elicits a cytotoxic T-cell response.

8. A therapeutic composition for ameliorating or delaying the onset of an inflammatory demyelinating disease comprising, in a pharmaceutically acceptable carrier, the recombinant pox virus of claim 1.

9. The therapeutic composition of claim 8, wherein the pox virus is selected from the group consisting of fowl pox, vaccinia and swine pox.

10. The therapeutic composition of claim 8, wherein the myelin protein is MBP or a variant thereof.

11. A method for generating an immune response to a myelin protein comprising,

a) introducing a sufficient amount of a first recombinant pox virus to a host to present the myelin protein to the immune system, wherein the first recombinant pox virus has at least one insertion site containing an operably linked DNA segment encoding the myelin protein ,

b) expressing the myelin protein intracellularly; and

c) presenting said myelin protein to the immune system

12. The method of claim 11 further comprising at least one periodic interval after introduction of the first recombinant pox virus and contacting the host with additional myelin protein or cytotoxic T-cell eliciting epitope thereof.

13. The method of claim 12, wherein the host is contacted with the additional myelin protein by introducing a second recombinant pox virus having at least one insertion site containing an operably linked DNA segment encoding the myelin protein.

14. The method of claims 13 where the myelin protein is

MBP or a variant thereof.

15. A method for presenting a myelin protein to the immune system in a host, comprising:

a. contacting the host with a sufficient amount of the myelin protein or cytotoxic T-cell eliciting epitope thereof; and

b. at least one periodic interval thereafter, contacting the host with additional myelin protein, wherein the host is contacted with the additional myelin protein by introducing a first recombinant pox virus having at least one insertion site containing an operably linked DNA segment encoding the myelin protein.

16. The method of claim 15, wherein the first recombinant pox virus is derived from a virus selected from the group consisting of suipox, avipox, capripox and orthopox virus.

17. The method of claim 16, wherein the pox virus is vaccinia, fowl pox or swine pox.

18. The method of claims 13 or 16, wherein the first

recombinant pox virus is derived from avipox and the second recombinant pox virus is derived from a pox virus selected from the group of pox viruses consisting of suipox, capripox and orthopox virus.

19. The method of claim 18, where either the first or second recombinant pox viruses are derived from an attenuated pox virus.

20. The method of claim 11 or 15, wherein the recombinant pox virus exhibits a low replicative efficiency in said host.

21. The recombinant vector of claim 1 which is vT92 or vT15.

FIG. 1A

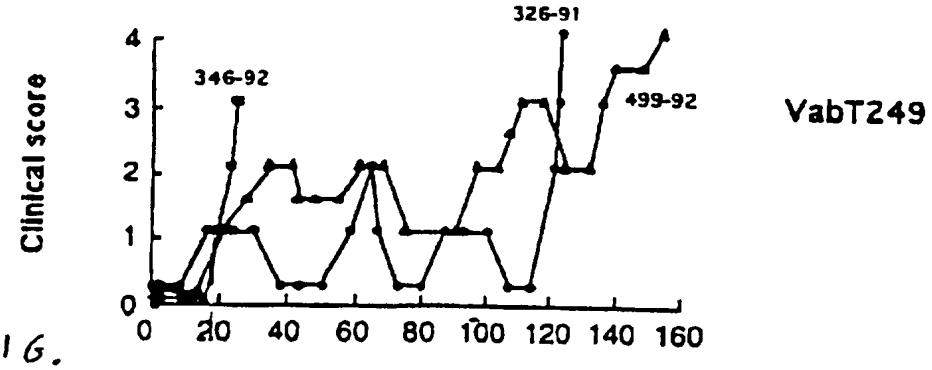

FIG. 1B

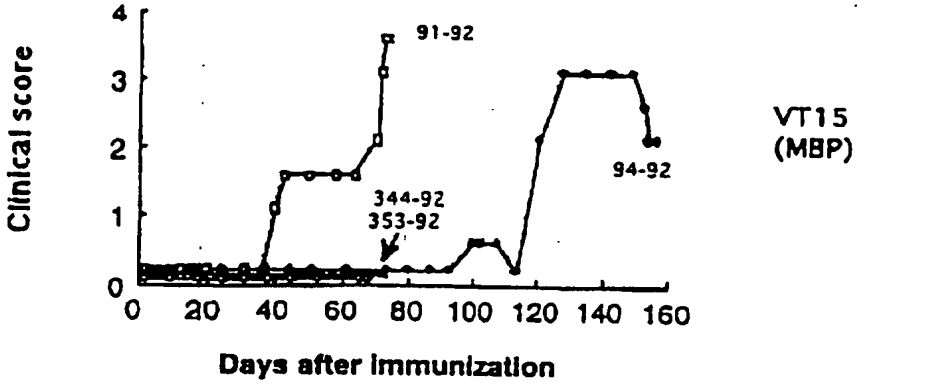

Figure 1.
Clinical course of EAE in vAbT249-(control) and vT15-(MBP) vaccinated C. *jacchus* marmosets.

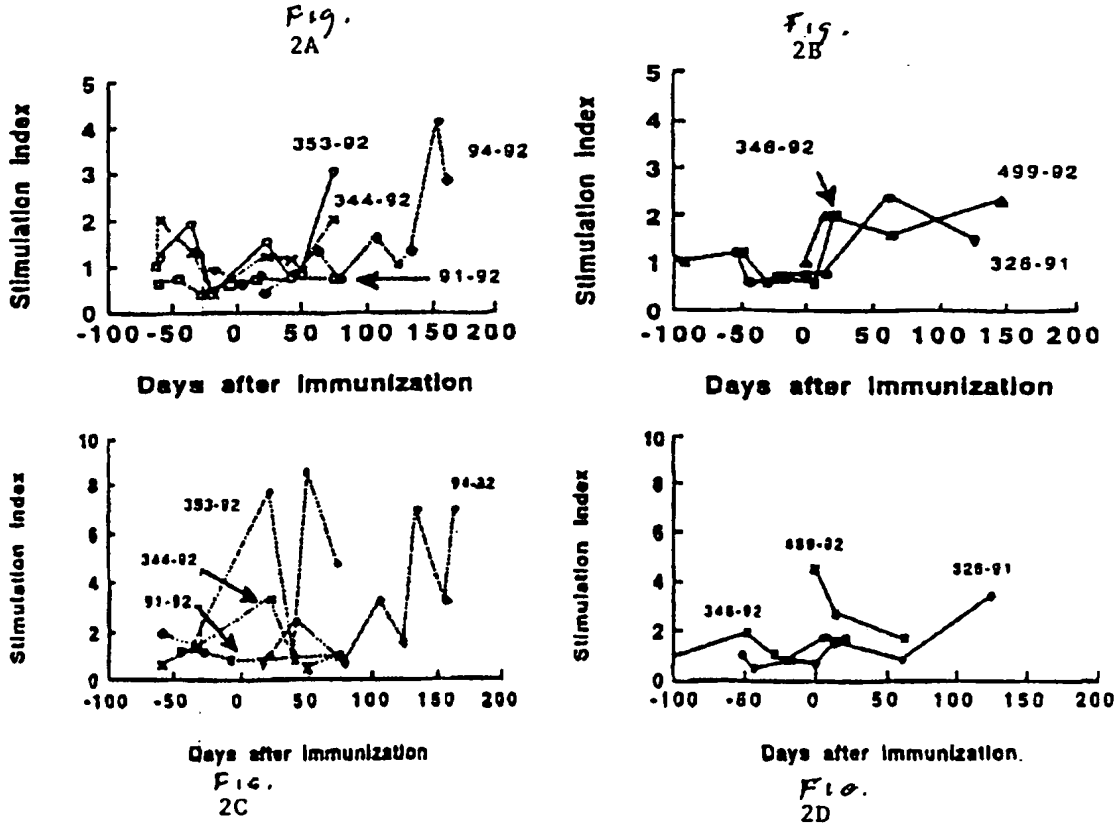

Figure 2

Proliferative responses in PBMC of WM-immunized C. *jacchus* marmosets vaccinated with vT15 (left) and vAbT249 (control, right) vaccinia virus. Top panels, in vitro stimulation wtih MBP (50 µg/ml); bottom panels, stimulation with NYCBH vaccinia antigen (2 µg/ml)

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(71) Applicants *(for all designated States except US)*: THERION BIOLOGICS CORPORATION [US/US]; 76 Rogers Street, Cambridge, MA 02142 (US). BETH ISRAEL DEACONNES MEDICAL CENTER [US/US]; 330 Brookline Avenue, Boston, MA 02215 (US). THE REAGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US).

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(74) Agents: EISENSTEIN, Ronald, I. et al.; Dike, Bronstein, Roberts & Cushman, L.L.P., 130 Water Street, Boston, MA 02109 (US).

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(57) Abstract

A recombinant pox virus encoding a myelin protein is described. The recombinant pox virus can be used for generating an immune response to the myelin protein. This method involves introducing a sufficient amount of the pox virus to present the myelin protein to the immune system, expressing this myelin protein and presenting the myelin protein to the immune system.

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INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 97/05217

A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEUROLOGY, vol. 46, no. 2 suppl., February 1996, pages a220-a221, XP002038649 GENAIN, C. P. ET AL.: "Inhibition of allergic encephalomyelitis in marmosets by vaccination with recombinant vaccinia virus encoding for Myelin Base Protein" see abstract ---	1-12,21
X	JOURNAL OF CLINICAL INVESTIGATION, vol. 96, no. 6, December 1995, pages 2966-2974, XP002038650 GENAIN, C.P. ET AL.: "Antiboby facilitation of Multiple Sclerosis-like lesions in a nonhuman Primate" see page 2973, column 1, line 4 - line 9 -----	1-5,8-10

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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2 .

Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Chambonnet, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/05217

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although as far as claims 11 - 20 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

CORRECTED VERSION*

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*(Referred to in PCT Gazette No. 04/1998, Section II)

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RECOMBINANT POX VIRUS ENCODING MYELIN PROTEIN FOR THERAPY

This invention was partially funded under NIH Grant No. NS30727 and the United States Government has certain rights thereto.

BACKGROUND OF THE INVENTION

Inflammatory demyelinating diseases of the central nervous system (CNS) such as multiple sclerosis (MS) and Guillian-Barre syndrome are believed to involve an immune response against CNS autoantigens. By analogy from disease models such as experimental allergic encephalomyelitis (EAE), indirect evidence suggests that an immune response against myelin proteins, particularly myelin basic protein (MBP), plays a role in the onset and progression of inflammatory demyelination disease. For example, in humans with MS, MBP-reactive T-cells can typically be recovered from fluids of MS patients [1, 2 and 3]. These T-cells are concentrated in the cerebrospinal fluid (CSF) of MS patients when compared to controls with other neurological diseases (2). MBP-reactive T cells have also been directly detected in MS lesions (3). In EAE, MBP is a major antigen since the disease can be induced either by active immunization with MBP, antigenic fragments of MBP, or by adoptive transfer of MBP-reactive T cells (4,5).

MBP is one component of myelin protein. The MBP gene encodes several MBP-related proteins through alternative RNA splicing (see Lemki, G. (1988), Mikoshiba, K., et al. (1991), Kamholz, J., (1987)). The gene sequences of other myelin proteins such as proteolipid protein (PLP), myelin-associated glycoprotein (MAG), and

myelin/oligodendrocyte glycoprotein (MOG) are also known [see Linington, et al. Eur J. Immunol. 23: 1364 (1993).

Animals that are susceptible to EAE are recognized models for the study of human inflammatory demyelinating diseases such as, e.g., MS. EAE can typically be induced by immunization with whole myelin, MBP, antigenic fragments of MBP, or by adoptive transfer of MBP-reactive T-cells [12, 13, 4, 5]. Rodents with EAE generally recover from the disease, indicating that the immune system suppresses the disease, e.g., by providing cytotoxic T-cells or anti-inflammatory cytokines [6-11]. Human MS however, is a relapsing and remitting disease which differs from most forms of acute rodent EAE. Little is known about the factors responsible for relapses, and the role of regulatory T-cells (e.g., T suppressor cells) in the human disease has not been thoroughly investigated.

In the primate *Callithrix jacchus* (*C. jacchus*, the common marmoset), EAE can be induced by active immunization with whole white matter (WM), MBP, or by adoptive transfer of MBP-reactive-T-cells or clones [12-14]. The marmoset is an especially advantageous model for the study of inflammatory demyelinating disease, in part because siblings share a bone marrow chimerism which permits adoptive transfer of lymphocytes between genetically distinct individuals. In marmosets with EAE, T-cell and antibody reactivity against MBP is detected at the onset of disease and can be observed in peripheral blood mononuclear cell (PBMC) preparations (13).

Viruses of the family *Poxviridae* (pox viruses) are useful as vectors for the delivery of foreign genes and gene products in many clinical and research settings. Pox viruses of the genus *Orthopoxvirus*, particularly vaccinia, are used for several reasons. Among these are: (a) its wide use in humans in the eradication of smallpox; (b) its ability to infect a wide range of cells, including professional antigen presenting cells, and express the inserted gene product (i.e. foreign gene product) in a manner that has the potential to be processed in the context of class I and/or class II MHC molecules; and (c) use as a recombinant vaccine in the treatment of certain tumors (Kantor, J. et al. (1992)).

A variety of other pox viruses have been useful as vectors for vaccines and the expression of foreign genes in cells, such as viruses of the genus *Avipoxvirus* such as, e.g., fowl pox, canary pox and viruses of the genus suipox such as swine pox.

It would be desirable to develop a vaccine which can induce immunological tolerance to nerve sheath proteins, particularly myelin proteins such as, e.g., MBP and MBP-related proteins.

SUMMARY OF THE INVENTION

We have discovered that by using a recombinant pox virus to intracellularly express a myelin protein or a T-cell eliciting epitope thereof, we can ameliorate or delay the symptoms of an inflammatory demyelinating disease in a vertebrate host. In particular, we have found that by intracellularly co-expressing the recombinant pox virus

and the myelin protein, the host immune system will thereafter substantially tolerate the myelin protein, thereby helping to ameliorate or delay the symptoms of the disease. By intracelluler expression, we mean that the process of transcription and translation of a protein occurs within the host cell. The protein, itself, may then be maintained in the cytoplasm, or transported to the nucleus, the cell membrane, other areas within the cell or into the extracellular space.

The recombinant pox virus has at least one insertion site containing a DNA segment encoding the myelin protein or a T-cell eliciting epitope thereof, which DNA segment is operably linked to a promoter capable of expression in the host. By the term "myelin protein" is meant those proteins which constitute the concentric oligodendrocyte wrapping around axons such as, e.g., MBP, PLP, MOP and MAG. A preferred myelin protein is MBP, MBP-related proteins, and T-cell eliciting epitopes thereof. Other proteins include MOP and MDP (myelodendritic protein).

In general, a preferred method of the present invention comprises introducing a sufficient amount of a first recombinant pox virus vector into a host to stimulate an immune response, and present the myelin protein to the immune systems. In some instances, one would want to contact the host with additional antigen at least once thereafter as a "boost". Preferably, one uses a first recombinant pox virus derived from a pox virus of a different genus for the "boost", e.g., first administer using a recombinant pox virus derived from an avipox, then boost with another derived from suipox. The first recombinant pox virus vector comprises a DNA segment which

encodes the myelin protein or T-cell eliciting portion thereof. The additional antigen as aforesaid may be added, e.g., by using a second recombinant pox virus from an immunologically distinct pox virus, typically from a different genus than the first pox virus.

Alternatively, or in conjunction with expression of myelin protein by the first recombinant pox virus, additional myelin protein or T-cell eliciting fragment thereof, may be added by contacting the host with the myelin protein formulated with an adjuvant or in a liposomal formulation or contacting the host with DNA encoding the myelin protein either as direct DNA or in an alternative viral vector.

The present invention also features a cell, preferably a homogeneous population of cells, which includes either the recombinant pox virus DNA or the recombinant pox virus, where the encoded myelin protein is expressed intracellularly in a sufficient amount to elicit an immune response against the myelin protein. The cell is preferably one which is capable of supporting virus or protein expression for at least 48 hours, such as, e.g., a mammalian, avian, reptilian, or insect cell line.

In another aspect, the present invention also features therapeutic compositions which include one or more recombinant pox virus disclosed herein where the virus(es) is provided in a pharmaceutically acceptable carrier. The therapeutic compositions can be administered to a mammal, preferably a human, as a means for ameliorating or delaying the onset of an inflammatory demyelinating disease such as, e.g., MS. The therapeutic compositions of the invention can be

administered alone or as adjuncts to known therapies for treating inflammatory demyelinating disease such as, e.g, Beta-interferon, adrenocorticotropic hormone (ACTH) and corticosteroids for the treatment of MS. Accordingly, the treatment of inflammatory demyelinating disease in, for example, mammals and in a domesticated animal such as, e.g., a dog, cat, horse, rabbit, mouse, pig, cattle, reptile, gerbil, bird, sheep, goat and the like, or a captive animal such as a primate (e.g., chimpanzees, etc.) is within the scope of the present invention.

The term " inflammatory demyelinating disease" means any immunological response which destroys, damages or removes the myelin sheath of nerve fibers. An inflammatory demyelinating disease in a mammal, particularly a human, can be identified by such damage.

The invention also features a method for generating an immune response to a myelin protein in a host, the method including:

a. contacting the host with a sufficient amount of the myelin protein or T-cell eliciting epitope thereof; and

b. at least one periodic interval thereafter, contacting the host with additional myelin protein or T-cell eliciting epitope thereof.

The invention also features a method of manufacturing a myelin protein capable of eliciting an immune response. The method includes the steps of:

a) synthesizing a recombinant pox virus, preferably an avipox such as fowl pox, or canary pox, a capripox, or a suipox such as swine pox, which virus includes an operably linked DNA segment encoding a

myelin protein, preferably MBP, MBP-related protein, or a T-cell eliciting epitope thereof;

b) introducing the recombinant pox virus into a mammalian host, preferably a human, under conditions which permit the infection of cells which support gene expression by the virus, preferably dermal cells or muscle cells; and

c) expressing and appropriately presenting the myelin protein intracellularly so that an immune response is elicited in the host.

The method is also useful for producing cells (or cell lysate thereof) which can be used to detect the onset of or evaluate the progression of an inflammatory demyelinating disease in a host. The cells appropriately present the myelin protein and are therefore recognized by T-cells isolated from the host. The killing by the T-cells can be detected by standard techniques such as, e.g., chromium release from cells labelled with radioactive chromium.

In another embodiment of the present invention, the method further comprises isolating the cells infected by the recombinant pox virus and using the cells, either as whole cells or cell fractions, as an immunogen for the production of antibodies, preferably monoclonal antibodies, which bind intracellularly expressed myelin protein in the infected cell. Of course, the antibody may also bind a T-cell eliciting epitope of the myelin protein. In accordance with conventional immunological techniques, such antibodies can readily be made by those skilled in the art.

In yet another embodiment of the present invention, the method

further comprises isolating cells from the infected host (or isolating the infected cells themselves), and using them, either as whole cells or cell fractions, to induce or stimulate an immune response in a second host to ameliorate or delay the onset of an inflammatory demyelinating disease in a mammal such as, e.g., a human.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 A and B shows two graphs which depict the clinical course of EAE in vAbT249 (control) and vT15 (MBP) vaccinated *C. jacchus* marmosets.

Figures 2 A, B, C and D shows four graphs which illustrate the proliferative responses in PBMC of WM-immunized *C. jacchus* marmosets vaccinated with vT15 (MBP) (Figures 2 A and C) and vAbT249 (control) (Figures 2B and D). Figures 2A and B show in vitro stimulation with MBP (50μg/ml). Figures 2C and D show stimulation with NYCBH vaccinia antigen (2μg/ml).

Detailed Description of the Invention

Viruses of the family *Poxviridae* are well known cytoplasmic viruses. Thus, genetic material expressed by recombinant pox viruses of the invention typically remains in the cytoplasm and does not have the potential for inadvertently integrating into host cell genes. Furthermore, because pox viruses have large genomes, they can readily be used to deliver a wide range of genetic material including multiple foreign genes (i.e., act as a multivalent vector).

Pox viruses disclosed herein are typically viruses of the family *Poxviridae*, preferably selected from the group consisting of avipox, capripox, orthopox, entomopox and suipox. Preferred avipox viruses include fowl pox, canary pox, pigeon pox, turkey pox, quail pox. An especially preferred avipox is fowl pox. Preferred orthopox viruses include vaccinia, cowpox, mousepox (ectromelia), rabbitpox, racoon pox, and monkey pox. An especially preferred orthopox is vaccinia. Preferably the suipox is swine pox, and the capripox is sheep or goat pox virus.

Where the host is a human, it is sometimes preferable to use a pox virus whose host range is restricted and does not include humans. Such a restriction can be natural such as by the use of avipox, capripox, entomopox or suipox in a human. Restriction can also be accomplished by attenuating (i.e. weakening) the pox virus whose host range is a human to a sufficient degree that pox virus will no be virulent. See for example, the NYVAC strain of vaccinia, U.S. Patent No. 5,494,807, herein incorporated by reference. See also the MVA strain in U.S. Pat. No. 5,185,146, herein incorporated by reference. The use of such attenuated pox viruses as vectors is particularly preferred in a host whose immune system is impaired.

Although less preferred than the pox viruses, other recombinant viruses (and DNA derived therefrom) are within the scope of the present invention. These viruses include those derived from herpes virus, polyoma viruses (e.g, SV40 and polyoma), adenoviruses, adeno associated viruses, as well as RNA viruses such as retroviruses and picornavirus such as polio virus, sindbis, Venezuelan equine

encephalitis. Other viruses include iridoviruses such as frog virus, and African swine fever virus.

One needs to select a parental virus that is not virulent for the putative host animal. For example, one can use a more attenuated virus, a non-replicating virus for that host, etc.

In preferred embodiments of the present invention, the recombinant pox virus exhibits a low replicative efficiency in the infected (i.e. target) cells of the host. Generally, replicative efficiency can be determined by conventional virological and cell culture techniques such as, e.g., infecting with virus and then quantitating the amount of viral progeny produced by the infected cell by titration on permissive cells (e.g. by viral plaque assay). The amount of progeny virus so produced can be expressed over the total number of cells used in the experiment. Preferred pox viruses used in accordance with the present invention preferably produce, on average, no more than about 10 productive (i.e., infectious) progeny per cell, more preferably, no more than about 1 productive progeny, still more preferably, no more than 0.1 progeny per cell. A productive progeny is one that will infect and replicate in a permissive host cell.

As a result of the low replication efficiency and the non-integrative, cytoplasmic nature of preferred pox viruses, the recombinant pox viruses made therefrom will not result in sustained replication and infection of other cells. Thus, the vector and transformed cells will not adversely affect cells in the host animal at locations distant from where the target cell is. Accordingly, more

virulent pox virus can be used to make the recombinant pox viruses of the present invention, provided that the virulence of the virus has been reduced by, e.g., selection or chemical or genetic mutagenesis so as to produce an attenuated strain of the virulent pox virus. See previously incorporated U.S. Pat. No. 5,494,807.

Although not wishing to be bound by theory we believe that the recombinant pox viral vector elicits a CD8+ response. The myelin encoded antigen specifically triggers the down regulation of myelin antigen-specific CD4 cells, perhaps through a CD8+ cell mediated response. Alternatively, there could be secretion of inhibitory cytokines by regulatory T-cells, or a combination of both.

Preparation of recombinant pox virus and vectors

Basic techniques for preparing recombinant pox viruses containing a heterologous DNA sequence encoding a foreign protein are well known in the art. For example, one method of preparing recombinant pox virus involves homologous recombination between the viral DNA sequences flanking the DNA sequence in a donor plasmid and homologous sequences present in the parental virus (Mackett, et al., *Proc. Natl. Acad. Sci. USA* 79:7415-7419 (1982)). See also methods for manufacturing recombinant fowlpox virus described in U.S. Patent No. 5,093,258, the disclosure of which is incorporated herein by reference.

Other techniques for making recombinant pox virus include using a restriction endonuclease site that is naturally present or artificially

inserted in the parental viral vector to insert the heterologous DNA. See U.S. Pat. No. 5,445,953, incorporated herein by reference.

Conventional recombinant DNA manipulations such as restriction enzyme digestion, ligation, transfection, transformation, agarose and polyacrylamide gel electrophoresis, DNA sequencing, electroporation are well known in the art and have been described (see e.g., Sambrook et al. in *Molecular Cloning: A Laboratory Approach* (1988); Ausubel et al. *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley Intersciences (1993)).

As an illustrative example, the heterologous DNA sequence to be inserted into a pox virus can be placed into a donor plasmid, bacteriophage or virus such as, e.g., an *E. coli* plasmid such as pBR322 or pUC19. In instances where a donor plasmid is chosen, the plasmid preferably includes an origin of replication (ORI), and one or more detectable markers such as, e.g., an antibiotic resistance gene (e.g., ampicillin or tetracycline) for propagation in *E. coli*. As is more fully described, infra, the heterologous DNA is typically flanked by DNA sequences which are homologous to the section of pox virus DNA flanking the insertion site.

Separately, and in addition, the heterologous DNA gene sequence to be inserted is ligated to a suitable promoter. The promoter-gene linkage is then operably positioned in the plasmid so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of pox DNA which is the desired insertion region. With a parental pox viral vector, a pox

promoter is generally used. The resulting recombinant plasmid is then used to transform *E. coli* where the cells are then propagated under selective conditions to reproduce the recombinant plasmid. The reproduced plasmid is then purified by standard means. Of course, other plasmids as well as a eukaryotic vector (e.g., SV40 propagated in green monkey cells) may be employed to propagate the heterologous DNA (See Sambrook et al. supra).

A preferred method of making a recombinant pox virus of the present invention is by inserting the heterologous DNA of the plasmid into a pox virus by genetic recombination. For example, the recombinant plasmid containing the heterologous DNA sequence to be inserted is transfected into a cell culture, e.g., chick embryo fibroblasts, along with the pox virus, e.g., fowl pox or swine pox. Recombination between homologous pox DNA in the plasmid and the viral genome respectively, results in a recombinant poxvirus modified by the presence of the promoter-gene construct in its genome. Preferably, the site of recombination is one which does not affect virus viability.

As noted above, the gene is inserted into a region (insertion region or site) in the virus which does not substantially affect virus viability of the resultant recombinant virus. By the term "virus viability" is meant the capability of the recombinant pox virus to produce infectious viral particles within about 48 hours in a permissive host cell. Of course, in some situations it may be desirable for the insertion to affect virus viability such as when the insertion attenuates the resulting recombinant pox virus. See discussion, *infra*.

If it is not desirable to substantially affect virus viability, the artisan can readily identify such regions by, for example, by testing pre-determined segments of virus DNA for regions that support genetic recombination without seriously affecting virus viability. One region that can readily be used and is present in many viruses is the thymidine kinase (TK) gene, a gene that has been found in virtually all pox virus genomes examined [leporipoxvirus: Upton, et al.(1986) (shope fibroma virus); capripoxvirus: Gershon, et al.(1989) (Kenya sheep-1); orthopoxvirus: Weir, et al.(1983) (vaccinia); Esposito, et al. (1984) (monkeypox and variola virus); Hruby, et al.(1983) (vaccinia); Kilpatrick, et al. (1985) (Yaba monkey tumor virus); avipoxvirus: Binns, et al. (1988) (fowlpox); Boyle, et al. (1987) (fowlpox); Schnitzlein, et al. (1988) (fowlpox, quailpox); entomopox (Lytvyn, et al., (1992).

In vaccinia, in addition to the TK region, other insertion regions which do not substantially affect virus viability include, for example, the HindIII M fragment.

In fowlpox, in addition to the TK region, other insertion regions include, for example, the BamHI J fragment [Jenkins, et al., (1991)] the *Eco*RI-*Hind*III fragment, *Eco*RV-*Hind*III fragment, *Bam*HI fragment and the *Hind*III fragment set forth in EPO Application No. 0 308 220 A1. [Calvert, et al., (1993); Taylor, (1988); Spehner, et al., (1990) and Boursnell, et al. (1990)].

In swinepox preferred insertion sites include the thymidine kinase gene region.

In addition to the requirement that the heterologous DNA be inserted into an insertion region, successful expression of the inserted DNA by the recombinant pox virus requires the presence of a promoter operably linked to the heterologous DNA, i.e., the heterologous DNA, promoter and pox virus are in a suitable spatial relationship which supports transcription of the heterologous DNA. The promoter must be placed so that it is located upstream from the DNA to be expressed. Promoters are well known in the art and can be readily selected depending on the host and the cell type desired. For example in pox viruses, promoters derived from pox viral promoters are used. They can be based upon native promoters such as the vaccinia 7.5K or 40K or fowlpox promoters such as FPV C1 or they can be artificial constructs containing appropriate pox sequences. Enhancer elements can also be used in combination with promoters to increase the level of expression, although enhancers need not always be located upstream of the inserted heterologous DNA. Furthermore, the use of inducible promoters such as, e.g., heat or metal inducible promoters, are also well known in the art and will be preferred in some instances.

It is not necessary that the pox vector encode all pox proteins. Rather, one needs only so much of a pox virus as is necessary for infecting the target cell and to permit expression of the heterologeous protein. This is sometimes referred to as corresponding to a sufficient portion of the pox genome for infection and expression.

A recombinant pox virus of the invention may optionally include a marker, such as β-galactosidase, CAT, neomycin or methotrexate resistance, whereby the target cells of the host may be detected (or

selected). The use of such a marker allows the skilled artisan to screen various viral vectors for those that are non-lytic or non-cytopathic in a particular target host cell. For example, the gene encoding β-galactosidase (*lacZ*) can be inserted into any recombinant pox virus disclosed herein, whereby the modified virus vector is then introduced into the target host cell and the production of β-galactosidase is measured. Expression of β-gal provides an indication of viral infectivity and gene expression.

IMMUNE RESPONSES

A specific immune response against a myelin protein or T-cell eliciting epitope thereof, can be generated by administering between about 10^5-10^9 pfu of a recombinant pox virus of the invention, to a host. More preferably about 10^7-10^9 pfu is used, although this amount may vary depending several factors such as, e.g., the particular host used. The preferred host is a mammal such as, e.g., a domesticated animal, a captive animal, or a human. In some instances, it is desirable to "boost" the presentation by administering additional antigen to the host. This may be one to three months later. There may also be at least a second "boost" preferably one to three months after the first boost. The myelin protein T-cell eliciting epitope thereof may be administered using the same pox virus vector or, more preferably the antigen is administered using a second pox virus vector from an antigenically unrelated pox virus, or alternatively, the antigen may be administered directly using, for example, an adjuvant or liposome in a pharmaceutically acceptable carrier. Cytokines, e.g., IL-2, IL-6, IL-12 may be used as biologic adjuvants and can be administered systemically to the host. Alternatively cytokines or co-stimulatory

molecules, e.g., B7.1, B7.2, can be co-administered via co-insertion of the genes encoding the molecules into the recombinant pox vector.

Adjuvants include, for example, RIBI Detox (Ribi Immunochemical), QS21 and incomplete Freund's adjuvant. Methods for making liposomes for administration are known.

T-Cells

T-cells that react against the epitope(s) of myelin protein or a T-cell eliciting epitope thereof can be obtained from peripheral blood mononuclear cells (PBMC) by standard methods. For example, PBMC can be separated by using Lymphocyte Separation Medium gradient (Organon Teknika, Durham, NC, USA) as previously described [Boyum, et al., *Scand J. Clin Lab Invest* 21: 77-80 (1968)]. Washed PBMC are resuspended in a complete medium, for example, RPMI 1640 (GIBCO) supplemented with 10% pool human AB serum (Pel-Freeze Clinical System, Brown Dear, WI, USA), 2mM glutamine, 100 U/ml penicillin and 100 μg/ml of streptomycin (GIBCO). PBMC at a concentration of about 2 x 10^5 cells in complete medium in a volume of, for example, 100 μl are added into each well of a 96-well flat-bottom assay plate (Costar, Cambridge, MA, USA). Protein antigen such as selected MBP peptides is then added into the cultures in a final concentration of about 50 μg/ml and incubated at 37°c in a humidified atmosphere containing 5% CO_2 for 5 days. After removal of the MBP or cytotoxic T-cell eliciting epitope thereof from the media, the cultures are provided with fresh human IL-2 (10U/ml) after 5 days and replenished with IL-2 containing medium every 3 days. Primary cultures are restimulated with the same peptide (50 μg/ml) on day 16. 5 x 10^5 irradiated (4,000

rad) autologous PBMC are added in a volume of about 50 μl complete medium as antigen-presenting cells (APC). About five days later, the cultures are provided with human IL-2 containing medium as described previously. Cells are restimulated for 5 days at intervals of 16 days.

Epitope mapping

T-cells prepared as described herein can be used to identify the epitope(s) of a myelin protein or fragment thereof, that are recognized by T-cells including cytotoxic T-cells. One method of preparing a T-cell eliciting epitope of a myelin protein includes limited proteolytic digestion of the protein with enzymes such as, e.g., papain, trypsin, or chymotrypsin (supplied by SIGMA Chemical Co. St. Louis, Mo.). Alternatively, fragments of a myelin protein can be chemically synthesized by conventional methods such as, e.g., the Merrifield Solid-Phase Technique. For example, cytotoxic T-cells can then be plated and the different myelin protein fragments added to different wells. Only cytotoxic T-cells that specifically recognize (i.e., bind) a peptide fragment with at least one epitope will continue to expand, thereby permitting ready identification.

T-cell eliciting epitopes of a myelin protein can be used as an alternative to using the entire protein. Additionally, one can prepare other fragments containing the epitope to enhance its ability to elicit a T-cell response.

Variants of Myelin Protein and DNA

Variants of myelin protein and DNA are within the spirit and scope of the present invention. For example, a degenerate variant of

the MBP DNA sequence is the same as that MBP sequence, except that the degenerate variant includes at least one nucleotide change which results in one or more alternative codons being used to encode the MBP amino acid sequence. The artisan will appreciate that there are 61 codons for the 20 common amino acids so that many of the amino acids are encoded by more than one (alternative) codon (see Darnell et al. eds., Scientific American Books, Inc., 1986).

Additional variants are within the spirit and scope of the present invention. For example, a recombinant pox virus of the invention may include the MBP DNA sequence, except that the variant will include at least one nucleotide change which results in one or more alternative codons being used to encode the MBP amino acid sequence, whereby the alternative codon encodes a conservative amino acid, i.e. an amino acid which can substitute for another amino acid because of similar characteristics. Examples of conservative amino acid substitutions include, e.g., valine for glycine, arginine for lysine, leucine for valine, serine for threonine, etc. Of course, a conservative amino acid substitution, by definition, will not substantially affect T-cell eliciting epitope(s) of MBP.

In yet another example of a variant, a recombinant pox virus of the invention may include the MBP DNA sequence, except that the variant will include at least one nucleotide change which results in one or more alternative codons being used to encode the MBP amino acid sequence, whereby the alternative codon encodes a non-conservative amino acid, i.e., an amino acid with different characteristics than the corresponding amino acid in the MBP amino acid sequence. In this

example, the non-conservative amino acid substitution does not substantially affect T-cell eliciting epitope(s) of MBP.

Alternatively, or in addition, the recombinant pox virus may include deletions of the MBP DNA sequence, or insertion of foreign DNA into the MBP DNA sequence, provided that the deletions and/or insertions do not materially affect a T-cell eliciting epitope(s) of MBP.

A T-cell eliciting epitope of MBP in a polypeptide fragment is another type of MBP variant.

Therapeutic Compositions

The recombinant pox viruses of the present invention can be administered to a suitable host using any acceptable route, including, for example, scarification and injection, e.g., intradermal, subcutaneous, intramuscular, intravenous or intraperitoneal.

A therapeutic composition of the present invention will suitably include one or more recombinant pox virus of the invention, which composition will typically be administered in unit dosage form to a suitable host in a sterile aqueous or non-aqueous solution, suspension or emulsion in association with a pharmaceutically-acceptable carrier such as physiological saline. Formulations for parenteral administration may also contain as common excipients polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated napthalenes and the like. (See generally *Remington's Pharmaceutical Sciences* (Mack Pub. Co., Easton, PA, 1980).

The therapeutic compositions of the invention may be employed as the sole active agent in a pharmaceutical or can be used in combination with other compounds and/or therapies which serve to treat inflammatory demyelinating disease.

The amount of the recombinant pox virus in the therapeutic composition will vary depending on a number of factors, including the dosage of the virus administered, the virulence of the selected virus, and the route of administration. In general terms, a composition is provided in aqueous physiological buffer solution containing about 10^5 to 10^9 pfu for parenteral administration. When the host is a human, typical dose ranges will include about 10^5 to 10^9 pfu recombinant avipox or suipox virus, preferably fowl pox or swine pox. However, the preferred dosage of the therapeutic composition is likely to depend on such variables as the type and extent of progression of the inflammatory demyelinating disease, the overall health status of the patient, the relative virulence and biological efficacy of the particular recombinant pox virus selected, the formulation of the composition excipients and its route of administration.

REFERENCE EXAMPLE 1
CONSTRUCTION OF VECTORS

Pox Viruses

A number of pox viruses have been developed as live viral vectors for the expression of heterologous proteins (Cepko et al., (1984); Morin et al., (1987); Lowe et al., (1987); Panicali & Paoletti, (1982); Mackett et al., (1982)). Representative fowlpox, swinepox virus and vaccinia are available through the ATCC under accession numbers VR-229, VR-363, and VR-325 respectively.

DNA Vectors For *In Vivo* Recombination With A Parent Virus

Genes that code for MBP or a T-cell eliciting epitope thereof, are inserted into the genome of a pox virus such as e.g., fowl pox virus, swine pox or vaccinia in such a manner as to allow them to be expressed by that virus along with the expression of the normal complement of parent virus proteins. This can be accomplished by first constructing a DNA donor plasmid for *in vivo* recombination with a pox virus.

In general, the DNA donor plasmid contains the following elements:

(i) a prokaryotic origin of replication, so that the vector may be amplified in a prokaryotic host;

(ii) a gene encoding a marker which allows selection of prokaryotic host cells that contain the vector (e.g., a gene encoding antibiotic resistance);

(iii) at least one gene encoding a desired myelin protein (e.g., MBP), each located adjacent to a promoter capable of directing the expression of the gene; and

(iv) DNA sequences homologous to the region of the parent virus genome where the foreign gene(s) will be inserted, flanking the construct of element (iii).

Methods for constructing donor plasmids for the introduction of multiple foreign genes into pox virus are described in W091/19803, the techniques of which are incorporated herein by reference. In general, all DNA fragments for construction of the donor vector, including fragments containing transcriptional promoters and fragments containing sequences homologous to the region of the parent virus genome into which foreign genes are to be inserted, can be obtained from genomic DNA or cloned DNA fragments. The donor plasmids can be mono-, di-, or multivalent (i.e., can contain one or more inserted foreign gene sequences).

The donor plasmid preferably contains an additional gene which encodes a marker which will allow identification of recombinant viruses containing inserted foreign DNA. Several types of marker genes can be used to permit the identification and isolation of recombinant viruses. These include genes that encode antibiotic or chemical resistance (e.g., see Spyropoulos et al.(1988); Falkner and Moss.(1988); Franke et al.(1985), as well as genes such as the *E. coli lac*Z gene, that permit identification of recombinant viral plaques by colorimetric assay (Panicali et al.(1986)).

Integration Of Heterologous DNA Into Pox Virus

Homologous recombination between donor plasmid DNA and viral DNA in an infected cell results in the formation of recombinant viruses that incorporate the desired elements. Appropriate host cells for *in vivo* recombination are generally eukaryotic cells that can be infected by the virus and transfected by the plasmid vector. Examples of such cells suitable for use with a pox virus are chick embryo fibroblasts, HuTK143 (human) cells, and CV-1 and BSC-40 (both monkey kidney) cells. Infection of cells with pox virus and transfection of these cells with plasmid vectors is accomplished by techniques standard in the art (Panicali and Paoletti, U.S. Patent No. 4,603,112, WO89/03429, both references herein incorporated by reference).

Following *in vivo* recombination, recombinant viral progeny can be identified by one of several techniques. For example, if the DNA donor vector is designed to insert one or more foreign genes into the parent virus thymidine kinase (TK) gene, viruses containing inserted DNA will be TK^- and can be selected on this basis (Mackett et al., (1982)). Alternatively, co-integration of a gene encoding a marker or indicator gene with the foreign gene(s) of interest, as described above, can be used to identify recombinant progeny. One preferred indicator gene is the *E. coli lacZ* gene: recombinant viruses expressing β-galactosidase can be selected using a chromogenic substrate for the enzyme (Panicali et al.(1986)).

Characterizing Antigens Expressed By Recombinant Pox Viruses

Once a recombinant pox virus has been identified, a variety of methods can be used to assay the expression of the polypeptide

encoded by the inserted gene. These methods include black plaque assay (an *in situ* enzyme immunoassay performed on viral plaques), Western blot analysis, radioimmunoprecipitation (RIPA), and enzyme immunoassay (EIA). Antibodies that recognize a myelin protein such as, e.g., MBP, MBP-related proteins, or T-cell eliciting epitopes thereof, are commercially available or, alternatively, can be made by conventional techniques. For example, monoclonal antibodies against these MBP, MBP-related proteins, or T-cell eliciting epitopes thereof, can be made by employing standard hybridoma technology (see, e.g., Kohler et al. (1975); Kohler et al. (1976); Hammerling et al. In *Monoclonal Antibodies and T Cell Hybridomas*, Elsevier NY, 1981; Harlow and Lane in *Antibodies: A Laboratory Approach* CSHSQB (1988); and Ausubel et al. supra).

Measurement of Antibody Titers

Prior to each immunization and 2 weeks following each immunization, anti-MBP antibody was quantified by ELISA. For example, microliter plates were coated with MBP (100 ng/well, SOURCE), ovalbumin (100 ng/well, Sigma), or $1x10^7$ PFU/well UV-inactivated V-Wyeth in PBS. The plates were blocked with 2% BSA in PBS, dried, and stored at -20° C until used. The plates were incubated with serum diluted 1:5, as well as a monoclonal antibody for PSA (DAKO M750, Denmark) as a standard control, for 24 hours at 4° C. Plates were washed several times with PBS containing 1% BSA, and incubated at 37° C for 45 min with horseradish peroxidase-conjugated goat anti-human IgG or IgM heavy chain specific antiserum (1:8000) (Southern Biotechnology Associates, Birmingham, AL) and antibody detected by HRP substrate system (Kirkegaard & Perry Laboratories,

Gaithersburg, MD) according to the manufacture's instructions. The absorbance of each well was read at 405 nm using a Bio-Tek EL310 microplate ELISA reader (Winooski, VT).

Optimizing immune responses to recombinant pox virus

Administration of a recombinant pox virus disclosed herein may, in some settings, elicit an undesirably strong immune response to the pox virus vector. Repeated administration of the same vector in subsequent boosts might result in undersirably rapid clearance of the virus from the host, not allowing sufficient time to express the myelin protein and boost the immune response to it. Thus while numerous "boosts" with recombinant pox virus of the invention are possible, repeated use of any one virus thereof may not always be preferred. The use of recombinant pox viruses from an antigenically distinct pox virus can in most cases minimize this problem.

In accordance with the present invention, to minimize high level immune response to a recombinant pox virus, e.g., when the first pox virus (or group of recombinant pox viruses) is fowl pox, the second and subsequent recombinant pox virus is selected from a different genus such as an orthopox, e.g. vaccinia, or a suipox.

Alternatively, another method of minimizing an undesirable immune response is to select an appropriate pox virus which exhibits a suitable host range and/or tissue specificity. For example, pox virus can be selected whose primary host range is different than the animal that the gene delivery system is to be used in. For example, avipox such as, e.g., fowl pox, or suipox such as, e.g, swine pox can be

used when the host is a non-permissive mammal such as a human. As an example, for those veterinary applications where the host is a pig, the use of suipox would not be advantageous. Accordingly, attenuated strains of pox viruses may be desirable when another pox virus outside the host range of the host is not available. For example, highly attenuated strains of certain orthopox viruses such as vaccinia (MVA strain) may be used or alternatively, may be further modified by conventional genetic or chemical mutagenesis techniques to be even more attenuated or non-virulent in the normal host range.

The cell specificity of the pox virus of interest is one way to easily screen for infectivity and replication efficiency.

EXAMPLE 1

Construction of recombinant fowlpox virus expressing MBP

The gene encoding MBP was excised from a 2.2 kb cDNA fragment isolated from a human brain cDNA library [Kamholz *et al.*, PNAS (USA) 83:4962-4966 (1986)]. A 1.2 kb DNA fragment, containing the entire 516 nucleotide coding sequence for MBP, 36 nucleotides of the 5' untranslated region, and 661 nucleotides of the 3' untranslated region, was inserted into a fowlpox virus plasmid vector. The resulting plasmid, designated pt3064, is used to make vT92, which contains the MBP gene under the control of the vaccinia virus 40K early/late promoter (Gritz *et al.*, 1990) and the *E. coli lacZ* gene under the control of the fowlpox virus C1 promoter (Jenkins *et al.*, 1991). This plasmid was deposited with the ATCC on March 22, 1996 and given accession number 97491. The foreign sequences are flanked by DNA sequences from the Bam HI J region of the fowlpox

virus genome (Jenkins *et al.*, 1991). A derivative of the POXVAC-TC (Schering Corp.) vaccine strain of fowl pox virus was used as the parental virus. The generation of recombinant fowlpox virus was accomplished via homologous recombination between fowlpox sequences in the parental fowlpox virus genome and the corresponding sequences in pT3064 in fowlpox-infected chick embryo dermal (CED) cells transfected with pT3064. Recombinant virus, designated vT92, was identified and plaque-purified by growth on CED cells in the presence of Bluogal (Life Technologies; Gaithersburg, MD), a chromogenic substrate for β-galactosidase. Insertion of the MBP gene into the fowlpox genome was confirmed by amplification of the inserted sequences by polymerase chain reaction (PCR). Expression of MBP was demonstrated by Western analysis using MBP-specific antisera.

Construction of recombinant vaccinia virus expressing MBP

The gene encoding for human MBP was derived from a 2.2 kb cDNA fragment isolated from a human cDNA library [21]. A 1.2 kb DNA fragment containing the 516 coding nucleotide sequence for MBP, 36 nucleotide of the 5' untranslated region, and 661 nucleotides of the 3' untranslated region, was inserted into a vaccinia virus transfer vector. The resulting plasmid, designated pT115, containing the MBP gene under the control of the vaccinia virus early/late promoter [22] flanked by DNA sequences from the Hind III M region of the vaccinia genome. These flanking sequences include the vaccinia K1L host range gene which is required for multiplication in human cells [23]. The plasmid was deposited with the ATCC on March 22, 1996 and given accession number 97490. A derivative of the New York City

Health (NYCBH) strain of vaccinia was used as the parental virus in the construction of recombinant vaccinia virus. This parental virus was designated vAbT33 (parent). This was deposited with the ATCC on May 15, 1989 and given ATCC No. VR-2240. The parental virus lacks a functional K1L gene and thus cannot efficiently replicate on rabbit kidney RK13 cells [24].

The generation of recombinant vaccinia virus was accomplished via previously described standard homologous recombination techniques. Briefly, homologous recombination was performed between vaccinia virus sequences in the vAbT33 (parent) genome and the corresponding sequences in RK13 cells infected with the parental virus and transfected with the pT115 vector. The RK13 cell line is publicly available (ATCC: Accession No. CCL37). A recombinant vaccinia virus bearing the MBP gene was selected by growth on RK13 cells. One recombinant vaccinia virus isolate included the entire MBP gene by Southern blot hybridization of restriction enzyme digests. The recombinant virus was designated vT15 (MBP). **Animals**

Marmosets (*C. jacchus)* were maintained in primate colonies at the University of California, San Francisco (UCSF). The animals used in this study were cared for in accordance with the guidelines of the Committee on Animal Research at UCSF and those of the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. A maximum of 2.5 ml of blood every other week was taken from each animal. Phlebotomy and immunization were done under brief anesthesia (ketamine, 10 mg intramuscularly).

Western Analysis of MBP Protein Expression

Expression of MBP was demonstrated in vT15(MBP) infected cell lysates by Western blot analysis using the polyclonal rabbit anti-guinea pig MBP R 120. This polyclonal antibody binds human MBP. The preparation of the antibody has been previously described ([25]. A control recombinant vaccinia virus was prepared which includes the gene encoding the equine herpes virus gH was prepared using homologous recombination as described above. The resulting recombinant vaccinia virus was designated vAbT249 (control).

Briefly, western experiments were conducted by infecting BSC-40 cells with either parental vaccinia virus (designated vAbT33) or the recombinant vaccinia virus (vT15 (MBP) in Dulbecco's Modified Eagle's Medium containing 2% fetal bovine serum. The infected cells were lysed in hypotonic lysis buffer (150 mM NaCl, 0.05% EDTA, 10 mM KCl, 1 mM PMSF) and then sonicated. Cell lysates and culture media were electrophoresed on an SDS-10% acrylamide gel. The proteins were transblotted to nitrocellulose, and the blot was incubated with a antibody specific for MBP (R120) at ambient temperature, washed, and then incubated with goat anti-rabbit phosphatase-labeled secondary antibody (AP, Kirkegaard & Perry Laboratories, Gaithersburg, MD) and developed according to the manufacturers instructions.

Vaccination with recombinant vaccinia viruses

Eight marmosets were vaccinated with about 10^7 pfu of either vAbT249 (control) or vT15 (MBP) in 4 subcutaneous injections in the back. In some animals, the initial injection was followed eight weeks later by a booster injection of 10^9 pfu. In a single monkey, we

observed generalized cowpox lesions and transient fever from 10-14 days after first vaccination. There were no other detectable side-effects over this time interval. The pathological and immunological consequences of vaccinating the animals were evaluated by measuring pathological manifestations such as, e.g., the appearance and severity of skin lesions, anti-vaccinia T-cell activity, and antibody responses as described fully below.

Induction of EAE in marmosets

21 to 35 days after vaccination with either vAbT249 (control) or vT15 (MBP), all animals were immunized with 100 mg human whole WM in complete Freund's adjuvant supplemented with 3 mg/ml H37Ra. On the day of immunization and again 48 hrs later, animals received intravenously 10^{10} killed *Bordetella pertussis* organisms (obtained from Lederle laboratories, NY). The onset and progression of EAE was monitored by daily clinical examination of the marmosets using standard pathologic criteria (see generally Massacesi, L., et al. (1995)). At the termination of experiments, animals were euthanized under ketamine anesthesia by exsanguination followed by a lethal dose of intravenous pentobarbital (20 mg/kg).

PBMC Infected with recombinant vaccinia virus intracellularly express MBP

Expression of MBP was monitored *in vitro* in Epstein-Barr virus-transformed B-lymphoblastic cell lines (B-LCL) infected with vT15 (MBP) or VAbT249 (control). MBP Expression was also monitored *in vivo* in PBMC every 2-3 weeks following vaccination. Cytospin preparations (Shandon) were fixed in 50% ethanol, blocked with 1%

bovine serum albumin in phosphate buffered saline and stained with R 120 rabbit polyclonal anti guinea pig MBP (1:200), after washing slides were incubated with peroxidase-conjugated anti rabbit IgG (Sigma). Color development was achieved using 3-amino-9-ethyl-carbazole (Sigma) and slides were counterstained with hematoxylin/eosin.

Briefly, expression of MBP in B-LCL cells was conducted in accordance with the following method: Monkey autologous B lymphoblastoid cell lines (BLCL) were established by infecting $1x10^5$ freshly isolated PBMCs in 100 ml of RPMI 1640 supplemented with L-glutamine, gentamicin, and 10% FCS (Biofluids, Rockville, MD) with 100 ml supernatant from S594 cells (kindly provided by Dr. M. D. Miller, Harvard Medical School, New England Regional Primate Research Center, Southborough, MA), which contains the baboon herpesvirus *Herpes papio*, in a 96 well, flat-bottomed plate (Costar, Cambridge, MA). Following transformation, cells were expanded, and media changed once weekly.

We found that recombinant vaccinia virus vT15 expressed the myelin protein in PBMC obtained from vaccinated animals. This was not seen with a control group of marmosets vaccinated with vAbT249 (control). In each animal, the period during which MBP was expressed varied between 15-45 days after the first or initial vaccination.

Immune responses against MBP and vaccinia antigens

T-cell responses were measured in a standard 72 hr. proliferation assay [13] using ^{3}[H]-thymidine incorporation in 10^5 freshly isolated PBMC plated in 96 well round bottom plates. Briefly, each plate contained in 200 μl/AIM V (Gibco-BRL) and one of the following: no

addition (control); MBP 50 μg/ml; Proteolipid protein (PLP) 10 μg/ml; PHA 2.5 μg/ml; WM 0.1% (wt/vol.); NYCBH vaccinia strain 2 μg/ml. Stimulation indices were calculated as the ratio of unstimulated to stimulated PBMC. The term "stimulation index" means the ratio of the amount of T-cell activity in PBMC that is detected in a biological sample exposed to a recombinant vaccinia virus of the invention to the amount of T-cell activity in PBMC not exposed to the virus. Animals were terminated by euthanasia; spleen and lymph nodes harvested and then tested in the T-cell proliferation assays.

The production of MBP and vaccinia virus binding antibodies were followed every 14-21 days using a dot-blot filtration apparatus according to the manufacturer's instructions (Biorad) [13]. Briefly, 250 ng MBP or 500 ng NYCBH strain were adsorbed on 0.45 μm nitrocellulose filter membranes (Biorad), blocked and incubated in succession with a) serial dilutions of marmoset sera; b) anti-monkey IgG conjugated to peroxidase (Sigma, 1:4,000 dilution); c) diaminobenzidine (Pierce) as substrate for color development.

The vT15 (MBP) recombinant vaccinia virus alleviates EAE

In control animals vaccinated with vAbT249 (control), EAE developed within about 14-18 days after immunization with WM (Table I and Figure 1A), in agreement with prior work [12, 13, 26]. One control animal did not develop clinical EAE but unexpectedly died from anesthesia at day 14 after immunization. Table I is shown below:

Table I. Day of onset of EAE and neuropathologic findings in C. *jacchus* marmosets vaccinated with vT15 and vAbT249 vaccinia virus and immunized with WM.

Animal number	Vaccination	Clinical signs (day pi)	Sacrifice (day pi)	Inflammation	Demyelination
94-92	vT15	97	156	+ +	+ +
91-92	(MBP)	37	73	+ + +	+ +
344-92	"	none	63	+	-
353-92	"	none	63	+	-
326-91	vAbT249	14	120	+ + +	+ + +
499-92	(control)	18	163	+ + +	+ +
346-92	"	17	22	+ + +	+ + +

Day pi: day after induction of EAE by active immunization with WM.

In animals vaccinated with the recombinant vaccinia virus expressing MBP (vT15 (MBP)), clinical manifestations of EAE were markedly delayed (Table I and Figure 1B). For example, in two animals (animal nos. 344-92 and 353-92) no clinical signs of EAE were noted over a period spanning 63 days.

Clinical and pathological EAE in the vT15-vaccinated animals were consistently delayed or suppressed compared to the vAbT249-vaccinated controls. In previous experience with immunization of over 30 animals with WM in adjuvant/*bordetella pertussis*, over 75% of monkeys developed disease within 14-28 days and all developed disease by day 60 after immunization. Thus, it is not likely that the delayed onset of EAE in the present study (37-120 days) were due solely to the individual susceptibility of the animals in the vT15(MBP)-vaccinated group. The data indicate that the protective effects of vT15 (MBP) vaccination were specifically mediated through expression of MBP and were not due to a nonspecific modulation of immune responses by exposure to vaccinia virus.

Neuropathological examination was performed on one vaccinated control animal (animal no. 346-92) at the time of acute EAE (22 days after immunization and 5 days after onset of EAE, see Figure 1A).. In addition, two animals vaccinated with vT15 (MBP) (animal nos. 344-92 and 353-92, see Figure 1B) were studied 63 days after the first immunization. In the control animal, we found pathology typical of acute EAE in *C. jacchus* [12, 13]. For example, in the CNS white matter we observed multiple areas of perivascular infiltration comprised of mononuclear cells and macrophages. These

manifestations were accompanied by prominent concentric demyelination and early gliosis. No involvement of gray matter structures was found. These pathological manifestations were identical to those previously reported for untreated *C. jacchus* immunized with WM in adjuvant, indicating that vaccination with the control vaccinia virus did not modify EAE. By contrast, in the two vT15-vaccinated animals studied at day 63 after immunization, scarce perivascular infiltration was found and was unaccompanied by demyelination (Figure 1, Table I). Accordingly, by both clinical and neuropathologic criteria, prior vaccination with recombinant MBP-vaccinia protected *C. jacchus* marmosets against EAE.

Despite being an FDA approved vaccine component, vaccinia virus in some instances can have encephalitogenic properties (Paoletti et al. (1993)). We therefore investigated whether vaccination with the NYCBH strain of vaccinia virus could by itself augment this EAE in *C. jacchus*.

Accordingly, two marmosets from each group vaccinated with the vAbT249 (control) or vT15 (MBP) recombinant vaccinia virus and then injected with white matter (WM) were followed for a period of 73-163 days after injection of the WM (see Table I and Figures 1A and B). In both groups, some animals developed severe clinical signs (grade 3 and above) resulting in death or requiring euthanasia (see animal nos. 91-92 (at 70 days); 94-92, 326-91 and 499-92 (each at 120 days)). In the control animals, disseminated, extensive infiltration of both white and gray matter structures accompanied by extensive demyelination were found (Table I). However, in two animals

vaccinated with vT15 (MBP), no such clinical manifestations were observed prior to sacrificing the animals (Figure 1B). These observations indicate that acute disseminated encephalomyelitis may follow some vaccinations with vaccinia virus. Reducing the dosage, using an even more attenuated strain of vaccinia or using an alternative pox vector such as an avipox or suipox are expected to avoid such occurrences.

Immune response against myelin and vaccinia antigens

1. T-cell responses

^{3}[H]-thymidine incorporation in response to stimulation with MBP, proteolipid protein (PLP) and vaccinia virus (NYCBH) was serially measured in PBMC beginning on the day of vaccination and until the time of death. The results are summarized in Figure 2.

T-cell reactivity against MBP: In two marmosets vaccinated with vT15 (MBP), transient and modest (i.e. stimulation index of 2) proliferative responses were observed prior to induction of EAE with WM (animal nos. 344-92 and 353-92, see Figures 2A and C). This result indicates that vaccination with the recombinant vaccinia virus presents the autoantigen to the immune system in vivo. Following active immunization with WM, T-cell proliferative responses against MBP were observed by days 15 and 22 in two of the vAbT249 (control) vaccinated animals and by day 62 in the third control monkey. However, T-cell proliferation against MBP did not develop in the vT15 (MAB) vaccinated monkeys up to 74 days after immunization. In one animal in this group (animal no: 91-92), T-cell responses against MBP

remained negative during the entire experiment. In no instance was T-cell reactivity against PLP detected in these experiments.

T-cell reactivity against vaccinia virus: Strong T-cell proliferative responses against NYCBH were observed in both the experimental and control groups following either vaccination or immunization with WM (see Figures 2C and D). This indicated that subcutaneous injection of the recombinant vaccinia viruses used herein efficiently stimulated cellular immune responses in *C. jacchus*. In animals later developing acute disseminated encephalomyelitis, anti-vaccinia T-cell responses increased.

T-cell reactivity against PLP: No animal exhibited T-cell reactivity against PLP in response to immunization with WM. However, one animal developing EAE exhibited a strong proliferative response against PLP.

Antibody responses

A) Antibodies against MBP: Serum antibody titers were not detected following vaccination with either the vT15 (MBP) or vAbT249 (control) recombinant vaccinia viruses. However, antibodies which bind MBP developed 14-21 days after immunization with WM in all animals.

b) Antibodies against vaccinia virus: Antibodies against the NYCBH strain of vaccinia virus were detectable 14-21 days following the first vaccination. This result indicates that, like T-cell responses, humoral immunity against vaccinia antigens had been induced by

vaccination.

Presentation of antigens by vaccinia virus vectors is achieved in part via the endogenous pathway of antigen presentation in association with MHC class I antigens. This could stimulate the proliferation of regulatory T-cells, in particular CD8+ T-cells. Without wishing to be bound to any particular theory, the alleviation of EAE observed in these experiments may be due to either immunological suppression of certain subsets of T-cells, or secretion of inhibitory cytokines by regulatory T-cells, or both. These protective mechanisms (or others) may be negated by the encephalitogenic properties of vaccinia virus.

Accordingly, to provide a recombinant pox virus with reduced encephalitogenic potential, the recombinant fowl pox virus disclosed above can be used in place of the recombinant vaccinia virus, in order to ameliorate or delay the onset of an inflammatory demyelinating disease in a mammal, particularly domesticated animals, captive animals, or humans.

It will be readily apparent that some of the above-described compositions and methods can be used as a kit suitable for clinical or veterinary use. Such a kit would include one or more recombinant pox virus of the invention in a pharmaceutically acceptable carrier, or cell or homogeneous population of cells which include the recombinant pox virus. In particular, the cells or homogeneous population of cells can be part of a diagnostic kit whereby the cells or homogeneous population of cells intracellularly express a myelin protein, preferably MBP, MBP-related protein, or a T-cell eliciting epitope thereof, which

cells or homogeneous population of cells are capable of binding T-cells derived from a host suspected of having or predicted to suffer from an inflammatory demyelinating disease as described herein. In accordance with conventional immunological technique, the binding between the T-cells and the cells or homogeneous cells disclosed herein can be detected.

All publications mentioned in the specification are indicative of the level of skill of those in the art to which this invention pertains. All publications are hereby incorporated by reference to the same extent as if each individual publication were specifically and individually stated to be incorporated by reference.

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Although the foregoing invention has been described in some detail by way of illustration and example for the purposes of clarity of understanding, one skilled in the art will easily ascertain that certain changes and modifications may be practiced without departing from the spirit and scope of the appended claims.

We claim:

1. A recombinant pox virus derived from a pox virus, where the recombinant pox virus has at least one insertion site containing an operably linked heterologous DNA sequence encoding a myelin protein, wherein the recombinant pox virus is capable of modulating a host immune response.

2. The recombinant pox virus of claim 1, wherein the heterologous DNA sequence encodes MBP or a variant thereof.

3. The recombinant pox virus of claim 1, wherein the pox virus is an orthopox, avipox or suipox virus.

4. The recombinant pox virus of claim 3, wherein the pox virus is selected from the group consisting of vaccinia, fowl pox, or swine pox.

5. The recombinant pox virus of claim 1, wherein the pox virus is wild-type or attenuated.

6. The recombinant pox virus of claim 1, wherein the immune response is a T-Cell response.

7. A cell infected with the pox virus of claim 1, wherein the cell expresses the myelin protein and elicits a cytotoxic T-cell response.

8. A therapeutic composition for ameliorating or delaying the onset of an inflammatory demyelinating disease comprising, in a pharmaceutically acceptable carrier, the recombinant pox virus of claim 1.

9. The therapeutic composition of claim 8, wherein the pox virus is selected from the group consisting of fowl pox, vaccinia and swine pox.

10. The therapeutic composition of claim 8, wherein the myelin protein is MBP or a variant thereof.

11. A method for generating an immune response to a myelin protein comprising,

a) introducing a sufficient amount of a first recombinant pox virus to a host to present the myelin protein to the immune system, wherein the first recombinant pox virus has at least one insertion site containing an operably linked DNA segment encoding the myelin protein ,

b) expressing the myelin protein intracellularly; and

c) presenting said myelin protein to the immune system

12. The method of claim 11 further comprising at least one periodic interval after introduction of the first recombinant pox virus and contacting the host with additional myelin protein or cytotoxic T-cell eliciting epitope thereof.

13. The method of claim 12, wherein the host is contacted with the additional myelin protein by introducing a second recombinant pox virus having at least one insertion site containing an operably linked DNA segment encoding the myelin protein.

14. The method of claims 13 where the myelin protein is

MBP or a variant thereof.

15. A method for presenting a myelin protein to the immune system in a host, comprising:

a. contacting the host with a sufficient amount of the myelin protein or cytotoxic T-cell eliciting epitope thereof; and

b. at least one periodic interval thereafter, contacting the host with additional myelin protein, wherein the host is contacted with the additional myelin protein by introducing a first recombinant pox virus having at least one insertion site containing an operably linked DNA segment encoding the myelin protein.

16. The method of claim 15, wherein the first recombinant pox virus is derived from a virus selected from the group consisting of suipox, avipox, capripox and orthopox virus.

17. The method of claim 16, wherein the pox virus is vaccinia, fowl pox or swine pox.

18. The method of claims 13 or 16, wherein the first

recombinant pox virus is derived from avipox and the second recombinant pox virus is derived from a pox virus selected from the group of pox viruses consisting of suipox, capripox and orthopox virus.

19. The method of claim 18, where either the first or second recombinant pox viruses are derived from an attenuated pox virus.

20. The method of claim 11 or 15, wherein the recombinant pox virus exhibits a low replicative efficiency in said host.

21. The recombinant vector of claim 1 which is vT92 or vT15.

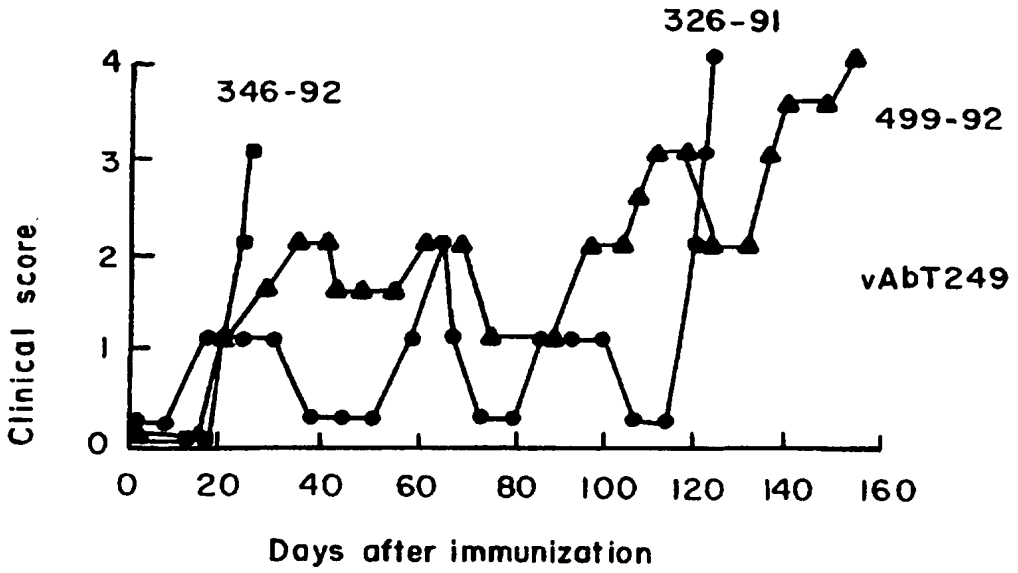
326-91
346-92
499-92
vAbT249
Clinical score
0
1
2
3
4
0 20 40 60 80 100 120 140 160
Days after immunization

FIG. 1A

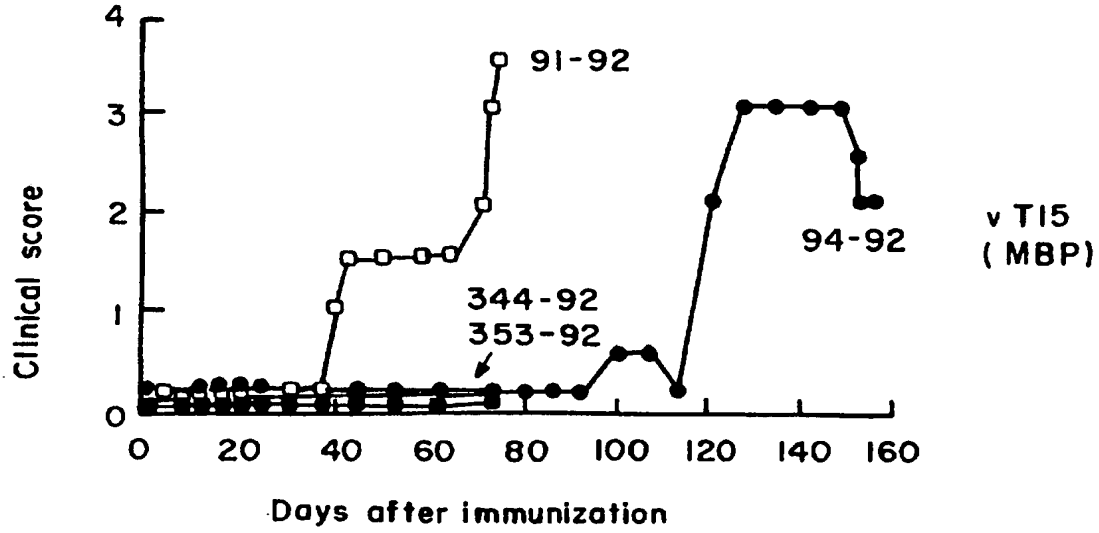
91-92
94-92
344-92
353-92
v T15
(MBP)
Clinical score
0
1
2
3
4
0 20 40 60 80 100 120 140 160
Days after immunization

FIG. 1B

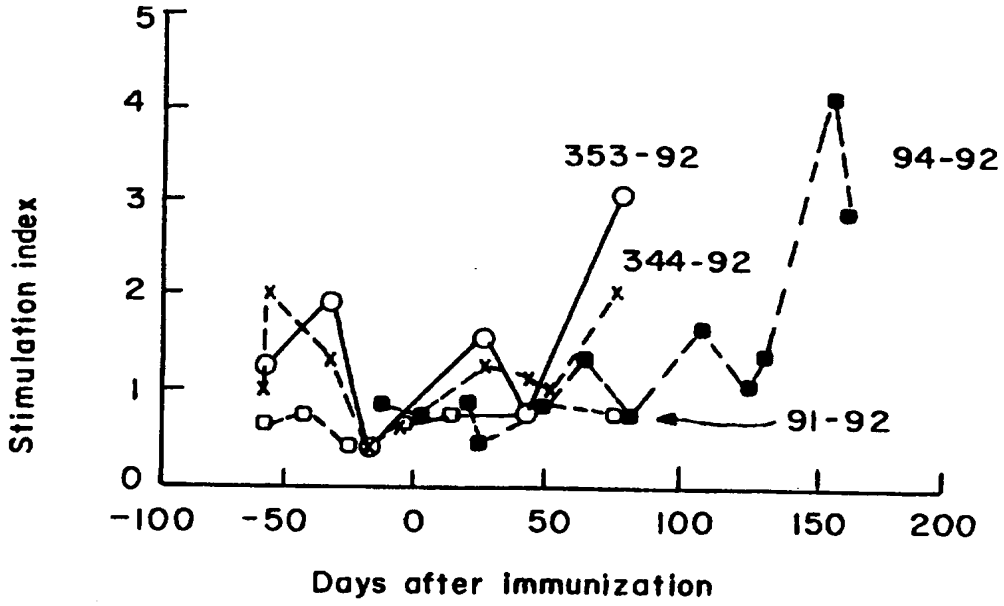
Stimulation index
5
4
3
2
1
0
353-92
94-92
344-92
91-92
-100
-50
0
50
100
150
200
Days after immunization

FIG.2A

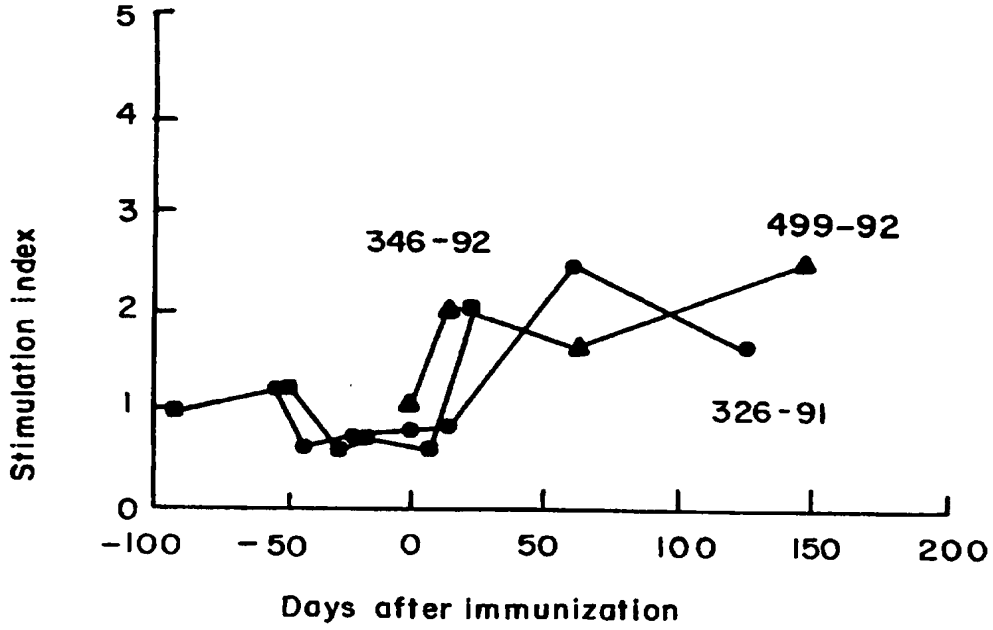
Stimulation index
5
4
3
2
1
0
346-92
499-92
326-91
-100
-50
0
50
100
150
200
Days after immunization

FIG. 2B

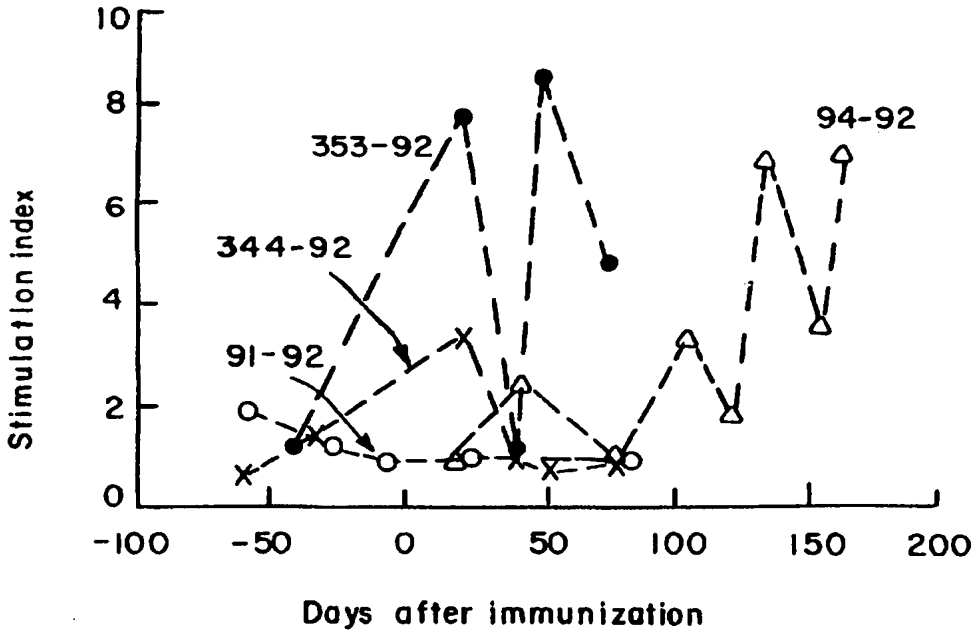
10
8
6
4
2
0
Stimulation index
353-92
94-92
344-92
91-92
-100
-50
0
50
100
150
200
Days after immunization

FIG. 2C

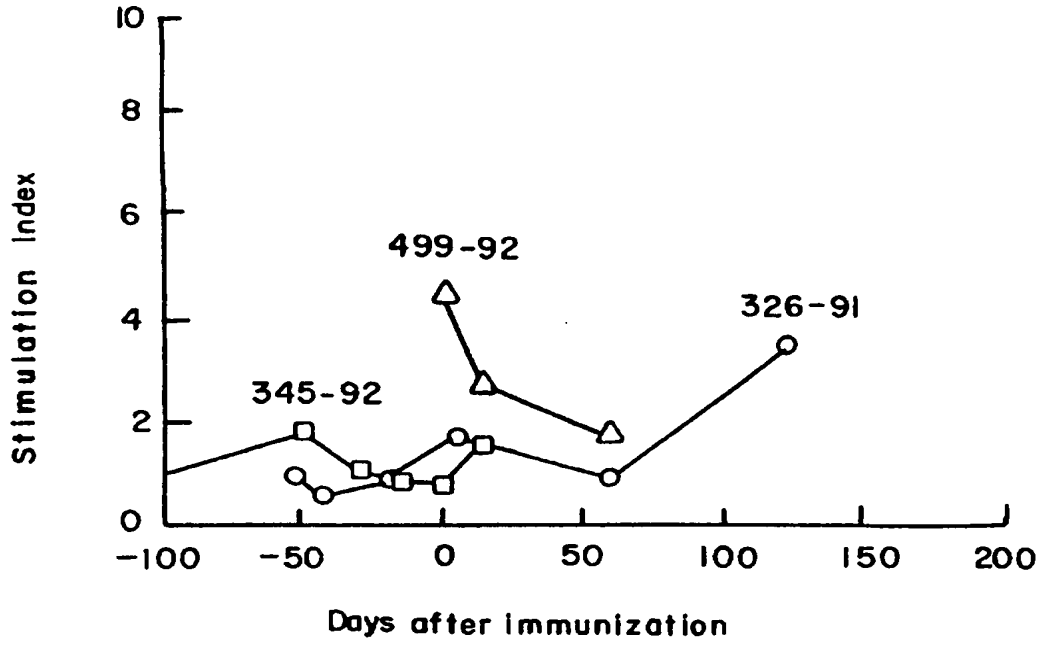
10
8
6
4
2
0
Stimulation Index
499-92
326-91
345-92
-100
-50
0
50
100
150
200
Days after immunization

FIG. 2D

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 97/05217

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C07K14/47 C12N5/10 A61K39/39 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEUROLOGY, vol. 46, no. 2 suppl., February 1996, pages a220-a221, XP002038649 GENAIN, C. P. ET AL.: "Inhibition of allergic encephalomyelitis in marmosets by vaccination with recombinant vaccinia virus encoding for Myelin Base Protein" see abstract ---	1-12,21
X	JOURNAL OF CLINICAL INVESTIGATION, vol. 96, no. 6, December 1995, pages 2966-2974, XP002038650 GENAIN, C.P. ET AL.: "Antiboby facilitation of Multiple Sclerosis-like lesions in a nonhuman Primate" see page 2973, column 1, line 4 - line 9 -----	1-5,8-10

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
22 August 1997	19.09.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Chambonnet, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/05217

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although as far as claims 11 - 20 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.